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Detection and molecular epidemiology of tick-borne encephalitis virus infections

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List of original publications

The thesis is based on the following original publications, which are referred to in the text by their Roman numerals. The copyright holders have given the permissions to reprint the articles.

I **Jääskeläinen A., Han X., Niedrig M., Vaheri A., Vapalahti O.** 2003. Diagnosis of tick-borne encephalitis by a μ-capture IgM-EIA based on secreted recombinant antigen produced in insect cells. Journal of Clinical Microbiology 41:4336-4342.

II **Jääskeläinen A. E., Tikkakoski T., Uzcátegui N. Y., Alekseev A. N., Vaheri A., Vapalahti O.** 2006. Siberian subtype tickborne encephalitis virus, Finland. Emerging Infectious Diseases 12:1568-1571.

III **Jääskeläinen A. E., Sironen T., Murueva G. B., Subbotina N., Alekseev A. N., Castrén J., Alitalo I., Vaheri A., Vapalahti O.** 2010. Tick-borne encephalitis virus in ticks in Finland, Russian Karelia, and Buryatia. Journal of General Virology 91:2706-2712.

IV **Jääskeläinen A. E., Tonteri E., Sironen T., Pakarinen L., Vaheri A., Vapalahti O.** 2011. European subtype tick-borne encephalitis virus in *Ixodes persulcatus* ticks. Emerging Infectious Diseases 17:323-325.

Abbreviations

ATCC	American type culture collection
bac-TBEV-prME	baculovirus-TBEV-prM and E construct
C	capsid protein
DENV	dengue virus
E	envelope protein
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
FSME	Frühsommer-Meningoenzephalitis (tick-borne encephalitis)
HI	hemagglutination inhibition assay
JEV	Japanese encephalitis virus
I.	*Ixodes*
IFA	immunofluorescence assay
IgG	immunoglobulin G
IgM	immunoglobulin M
kb	kilobase
kDa	kilodalton
LIV	louping ill virus
M	membrane protein
MAb	monoclonal antibody
NCR	non-coding region
NS	non-structural
nt	nucleotide
NT	neutralization test
ORF	open reading frame
PCR	polymerase chain reaction
prM	pre-membrane protein
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBE	tick-borne encephalitis
TBEV	tick-borne encephalitis virus
TBEV-Eur	tick-borne encephalitis virus, European subtype
TBEV-FE	tick-borne encephalitis virus, Far Eastern subtype
TBEV-Sib	tick-borne encephalitis virus, Siberian subtype
WNV	West Nile virus
YFV	yellow fever virus

Abstract

Tick-borne encephalitis (TBE) is a potentially severe central nervous system infection endemic in many European and Asian countries. The causative agent of the disease is a tick-transmitted flavivirus, tick-borne encephalitis virus (TBEV). There are three subtypes of the virus: European (TBEV-Eur), Siberian (TBEV-Sib) and Far Eastern (TBEV-FE). The vectors for TBEV are *Ixodes ricinus* (sheep tick) and *I. persulcatus* (taiga tick).

We developed an IgM enzyme immunoassay based on secreted recombinant prM and E antigen produced in insect cells for diagnosis of acute TBE. The test proved sensitive and specific and is now in use at the diagnostic laboratory of Hospital District of Helsinki and Uusimaa, Finland. We also studied the prevalence and molecular epidemiology of TBEV in Finland and Russia, and old as well as emerging TBE foci in Finland. The epidemiology of TBE depends on (micro)climatic and ecological factors, and based on predicted climate change it is likely that TBE will become more prevalent in northern Europe, including Finland.

In Finland the disease has been endemic in the Åland Islands, archipelagos of Turku, Helsinki, and Kokkola, and in Lappeenranta region. New endemic foci have appeared recently, including the world's northernmost TBE-endemic focus in Simo in northern Finland. We isolated seven TBEV-Eur strains from *I. ricinus* ticks and human sera from the southern regions of the country. The Finnish TBEV-Eur strains showed small-scale geographical clustering which supports the hypothesis that the endemic foci are maintained independently without the need of introducing new viral strains each summer. Unexpectedly, we found the western coast being inhabited by *I. persulcatus* ticks, which carry TBEV-Sib in Kokkola archipelago but unorthodoxly, TBEV-Eur in Simo. We isolated 11 TBEV-Sib strains from *I. persulcatus* from Kokkola archipelago. From Simo in Finnish Lapland we isolated six TBEV-Eur strains, two from *I. persulcatus* and four from bank voles. *I. persulcatus* has distributed to Simo within the last 50 years, and the establishment of a new focus by unusual combination of virus subtype and vector species indicates different dispersal routes of the virus and its vector.

We also studied ticks from two republics in the Russian Federation, Karelia in the north-west and Buryatia in eastern Siberia, and isolated two TBEV-Sib strains and one TBEV-FE strain, respectively, from *I. persulcatus*. The TBEV-Sib strains isolated from Finland and Russian Karelia belonged to the "Baltic" lineage of the Siberian subtype.

Altogether, we studied 3972 ticks. The prevalence of TBEV in ticks was about 1% in most of the studied endemic foci within a large geographical area. We characterized the Finnish TBE foci and found a new tick species for Finland, *I. persulcatus,* distributed in western Finland, carrying both TBEV-Eur and TBEV-Sib subtypes.

Review of the literature

Introduction

Tick-borne encephalitis (TBE) is a severe central nervous system infection. The causative agent is a flavivirus tick-borne encephalitis virus (TBEV), which is transmitted to humans and other warm-blooded animals by a bite of its vector, a tick. TBEV was isolated for the first time in 1937 from Soviet Union [Silber and Soloviev, 1946]. TBE is endemic in several European and Asian countries.

Taxonomy of flaviviruses

TBEV belongs to the genus *Flavivirus* of the family *Flaviviridae*. The family *Flaviviridae* includes two other genera as well: pestiviruses (border disease virus, bovine viral diarrhea viruses 1 and 2, classical swine fever virus, and a tentative species of the genus, pestivirus of giraffe), and hepaciviruses, which includes the hepatitis C virus clades, and, tentatively, GB virus [Thiel *et al.,* 2005]. Viruses in the separate genera of *Flaviviridae* have diverse biological properties but share common characteristics in virion morphology and RNA organization and replication [Lindenbach *et al.,* 2007].

Members of the genus *Flavivirus* are small (diameter about 50 nm) enveloped, spherical animal viruses whose genome is an about 11 kb single-stranded RNA of positive polarity. Most are arthropod-borne. In addition to *Ixodes* tick transmitted TBEV, flaviviruses include several other important human pathogens, e.g. *Aedes* mosquito transmitted dengue (DENV) and yellow fever (YFV) viruses and *Culex* mosquito transmitted Japanese encephalitis (JEV) and West Nile (WNV) viruses (Figure 1).

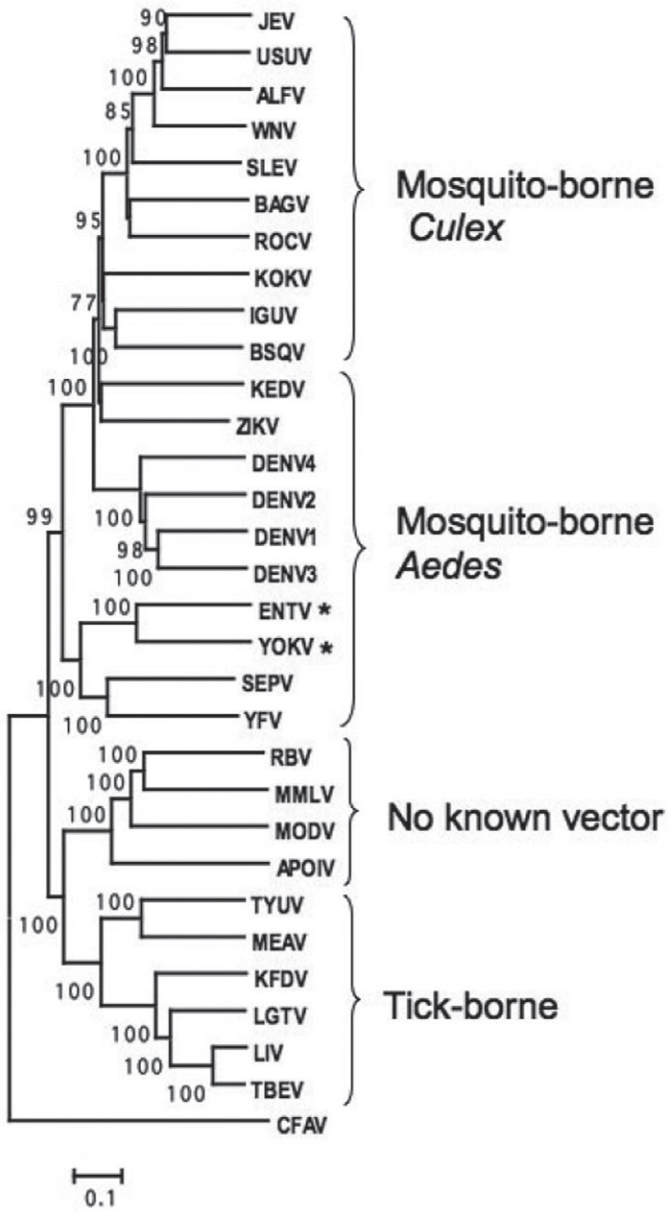

Figure 1. Neighbor-joining phylogenetic tree of complete open reading frame nucleotide (nt) sequences of 31 flaviviruses. Bar represents nt substitutions / site. *) No vector is known for Entebbe bat (ENTV) and Yokose (YOKV) viruses despite their grouping within the mosquito-borne flaviviruses. JEV, Japanese encephalitis virus; USUV, Usutu virus; ALFV, Alfuy virus; WNV, West Nile virus; SLEV, St. Louis encephalitis virus; BAGV, Bagaza virus; ROCV, Rocio virus; KOKV, Kokobera virus; IGUV, Iguape virus; BSQV, Bussuquara virus; KEDV, Kedougou virus; ZIKV, Zika virus; DENV1-4, dengue virus serotypes 1-4; SEPV, Sepik virus; YFV, yellow fever virus; RBV, Rio Bravo virus; MMLV, Montana myotis leukoencephalitis virus; MODV, Modoc virus; APOIV, Apoi virus; TYUV, Tyuleniu virus; MEAV, Meaban virus; KFDV, Kyasanur Forest disease virus; LGTV, Langat virus; LIV, louping ill virus; TBEV, tick-borne encephalitis virus; CFAV, cell-fusing agent virus. Figure courtesy of Dr. Eili Huhtamo [Huhtamo, 2010], with permission.

According to the latest report - from 2005 - of the International Committee on the Taxonomy of Viruses, there are 68 flavivirus species: 39 are transmitted by mosquitoes and 13 by ticks while the rest have no known arthropod vector [Thiel *et al.,* 2005].

Tick-borne flaviviruses are monophyletic and can be grouped to those associated with mammals and ticks that prefer feeding on mammals, and those associated with seabirds and their ticks [Thiel *et al.,* 2005]. The seabird-associated tick-borne flaviviruses are Kadam virus, Meaban virus, Saumarez Reef virus, and Tyuleniu virus. Mammalian tick-borne flaviviruses are listed in Table 1.

Table 1. Mammalian tick-borne flaviviruses.

Virus / subtype	**Abbreviation**	**Tick vector**	**Distribution**	**Disease association**
Tick-borne encephalitis virus, European subtype	TBEV-Eur	*Ixodes ricinus*	Europe	encephalitis
Tick-borne encephalitis virus, Siberian subtype	TBEV-Sib	*I. persulcatus*	Eurasia	
Tick-borne encephalitis virus, Far Eastern subtype	TBEV-FE			
Louping ill virus	LIV	*I. ricinus*	Europe	encephalitis in sheep and goats; encephalitis in humans rare
Spanish sheep encephalitis virus	SSEV			
Greek goat encephalitis virus	GGEV			
Turkish sheep encephalitis virus	TSEV			
Omsk hemorrhagic fever virus	OHFV	*Dermacentor reticulatus*	Siberia	hemorrhagic fever
Kyasanyr forest disease virus	KFDV	*Haemaphysalis spinigera*	India, China[1]	hemorrhagic fever
Alkhurma (Alkhumra) hemorrhagic fever virus	AHFV	*Ornithodoros savignyi*[2]	Saudi Arabia, Egypt	hemorrhagic fever
Powassan virus	POWV	*I. cookei*	North America	encephalitis
		H. longicornis, I. persulcatus	East Asia	
Deer tick virus	DTV	*I. scapularis*	North America	encephalitis
Gadgets Gully virus	GGYV	*I. uriae*[3]	Australia	not known
Langat virus	LGTV	*I. granulatus*	Malaysia	encephalitis
		H. papuana	Thalland	
		I. persulcatus	Siberia	
Royal Farm virus	RFV	*Argas hermanni*[4]	Afghanistan[4]	not known
Karshi virus	KSIV	*O. papillipes*[5]	Uzbekistan[5]	not known

Mammalian tick-borne flaviviruses, their principal tick vector species, endemic areas and disease associations. Virus species names according to International Committee on Taxonomy of Viruses [Thiel *et al.,* 2005] in bold, however, classification of species and subtypes may undergo changes in the future [Grard *et al.,* 2007]. Data collected from [Thiel *et al.,* 2005; Grard *et al.,* 2007; Gould and Solomon, 2008; Dobler, 2010; Lasala and Holbrook, 2010], and [1][Wang *et al.,* 2009], [2][Charrel *et al.,* 2007], [3][Major *et al.,* 2009], [4][Williams *et al.,* 1972], [5][Lvov *et al.,* 1976].

Tick-borne encephalitis virus has three subtypes: European, Siberian, and Far-Eastern, abbreviated as TBEV-Eur, TBEV-Sib, and TBEV-FE, respectively [Ecker *et al.,* 1999; Thiel *et al.,* 2005]. TBEV-Sib can be further divided to the "Siberian lineage" comprising TBEV-Sib strains isolated from Siberia, and the "Baltic lineage" consisting of TBEV-Sib strains isolated from the vicinity of the Baltic Sea, Estonia, Latvia, and Finland [Golovljova *et al.,* 2008]. All TBEV strains are closely related to each other. Within one subtype the variation at the amino acid level of the E protein is maximally 2.2%, and between the subtypes 3.6-5.6% [Ecker *et al.,* 1999].

The principal vector for TBEV-Eur is *Ixodes ricinus,* the sheep tick, and for TBEV-Sib and TBEV-FE, *I. persulcatus,* the taiga tick [Mavtchoutko *et al.,* 2000; Lundkvist *et al.,* 2001; Charrel *et al.,* 2004]. TBEV has occasionally been isolated in several other tick species and cultured in laboratory in other ticks and tick cell lines [Süss, 2003; Kim *et al.,* 2008, 2009; Ruzek *et al.,* 2008] but *I. ricinus* and *I. persulcatus* are the ecologically and epidemiologically significant vectors [Labuda and Randolph, 1999; Heyman *et al.,* 2010]. Within the tick-borne encephalitis virus subtypes, as among flaviviruses in general, phylogeny is rather well congruent with vector species (Figures 1 and 2). TBEV-Eur is more closely related to other *I. ricinus* transmitted flaviviruses causing encephalitis in sheep and goats i.e. louping ill virus (LIV), Turkish sheep encephalitis virus (TSEV), Spanish sheep encephalitis virus (SSEV), and Greek goat encephalitis virus (GGEV), than to *I. persulcatus* transmitted TBEV-Sib or TBEV-FE [Grard *et al.,* 2007]. The current taxonomy [Thiel *et al.,* 2005] is based on the biology of the viruses and thus the sheep- and goat-infecting TSEV, GGEV, and SSEV are considered subspecies of LIV and the human pathogen TBEV a separate species. However, when more and more sequences become available, grouping based on disease associations alone seems inadequate. For example, based on complete amino acid sequences, Grard and others (2007) suggested only one virus species called tick-borne encephalitis virus consisting of Western TBEV (including TBEV-Eur), Eastern TBEV (including TBEV-Sib and TBEV-FE), TSEV (including TSEV and GGEV) and LIV (including LIV and SSEV) [Grard *et al.,* 2007].

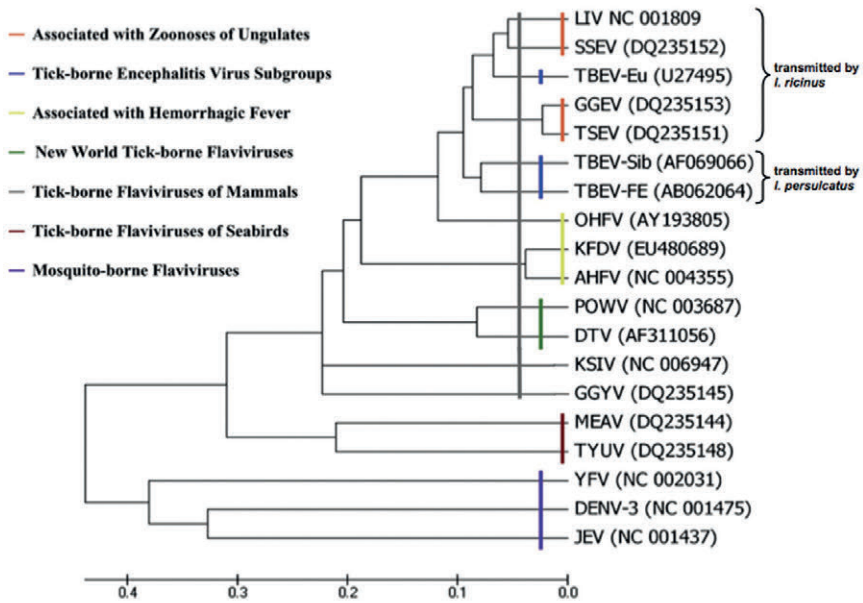

Figure 2. Phylodendogram of 9929 nt of 19 flaviviruses. GenBank accession numbers are shown. Biological similarities are indicated. Abbreviations of virus names are the same as listed in Figure 1 and Table 1. Modified from [Lasala and Holbrook, 2010].

Structure and replication

Flavivirus genome is an about 11 kb single-stranded RNA of positive polarity, thus, once released to the cytoplasm, it serves directly as a messenger RNA. The single open reading frame is translated to a large polyprotein which is co- and post-translationally processed (Figure 3). Host- and virus-encoded proteases cleave the polyprotein precursor to individual proteins [Lindenbach and Rice, 2003].

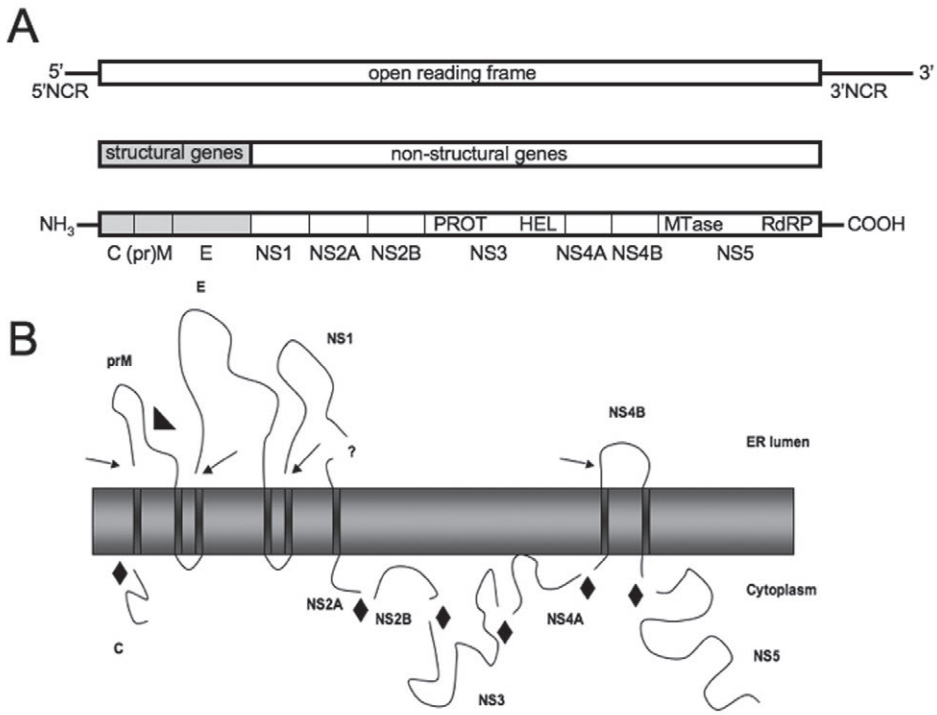

Figure 3. A. Genomic organization in flaviviruses. NCR: non-coding region; PROT: protease; HEL: helicase; MTase: methyltransferase; RdRP: RNA-dependent RNA polymerase. Modified from [Lindenbach *et al.,* 2007]. **B. Flavivirus polyprotein processing.** Polyprotein translated from the flavivirus ORF is co- and posttranslationally cleaved by virus-encoded serine proteases (diamonds), host-encoded signalases (arrows), or by furin (triangle). Figure courtesy of Suvi Kuivanen.

The genome is packaged inside the capsid, which is formed by capsid protein C and has an icosahedral symmetry. This nucleocapsid is surrounded by an envelope which consists of cell-derived lipid bilayer and E (envelope) and M (membrane) proteins. There are 180 copies of C protein in the capsid and M and E proteins on the surface of a flavivirus particle [Perera *et al.,* 2008; Sanchez-San Martin *et al.,* 2009]. When infecting a new cell, the viral and cell membranes fuse to release the viral nucleocapsid into the cytoplasm. Viral fusion protein, the E protein in the case of flaviviruses, is responsible for the fusion.

Most viral fusion proteins have been grouped to class I, II or III [Harrison, 2008], but some experts find the grouping confusing and too simplified and prefer not to use it [Harrison, 2008; Sanchez-San Martin *et al.,* 2009]. Class I fusion proteins (at least orthomyxo-, paramyxo-, retro- and filoviruses), e.g. the influenza virus hemagglutinin, form spiky projections out from the viral envelope. They are synthesized as precursors and need to be proteolytically cleaved to become active [Harrison, 2008]. Class II fusion proteins (flavi- and alphaviruses) lie flat on the virion surface and are folded together with another protein that

acts as a chaperone, and it is the chaperone rather than the fusion protein itself which is activated by proteolytic cleavage [Harrison, 2008; Sanchez-San Martin *et al.,* 2009]. For class III (herpes simplex virus I, rhabdoviruses), conformational changes induced by low pH are reversible and viruses can be reactivated by rising pH [Harrison, 2008].

The major envelope protein of flaviviruses, E protein, contains the fusion peptide responsible for fusion of the membranes of the virus and the cell. E is the major antigen and induces formation of protective antibodies [Chambers *et al.,* 1990], has hemagglutinating activity, and carries the receptor-binding sites needed for recognizing and attaching to a target cell.

Flavivirus cellular receptors vary according to virus species and cell type [Lindenbach *et al.,* 2007; Kaufmann and Rossmann, 2011] and not all are known. E.g. mannose-binding molecules have been suggested, including DC-SIGN (dentritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [Kaufmann and Rossmann, 2011] and mannose receptors on surfaces of macrophages for dengue viruses [Miller *et al.,* 2008], and for TBEV and JEV [Kaufmann and Rossmann, 2011]. Glycosaminoglycans, e.g. heparan sulfate proteoglycan, are apparently involved as well [Kroschewski *et al.,* 2003; Lindenbach *et al.,* 2007]. Flaviviruses may also infect macrophages and other cells having Fc-receptors by antibody-dependent enhancement in the presence of sub-neutralizing levels of antibody [Halstead, 2003; Takada and Kawaoka, 2003; Lindenbach *et al.,* 2007].

In a mature flavivirus virion ready to infect its target cell E protein molecules are organized as dimers lying flat on the surface of the virion [Heinz and Allison, 2001]. After E protein receptor-binding site(s) have bound to cellular plasma membrane receptors, the virus enters the cell by clathrin-dependent receptor-mediated endocytosis [Chambers *et al.,* 1990; Lindenbach and Rice, 2003; Mukhopadhyay *et al.,* 2005; Kaufmann and Rossmann, 2011]. The mildly acidic pH (6.6-6.8 [Perera *et al.,* 2008]) inside the endosomes triggers irreversible conformational changes in the E protein molecules. They reorganize to protruding trimers so that the fusion peptide, which is hidden at neutral pH, becomes exposed. Now the hydrophobic exterior of the fusion peptide allows insertion into the target membrane [Seligman and Bucher, 2003]. This leads to fusion of the virus and endosomal lipid bilayers and release of the flavivirus RNA to the cytoplasm [Kaufmann and Rossmann, 2011]. The details of nucleocapsid uncoating are not fully understood [Lindenbach and Rice, 2003; Kaufmann and Rossmann, 2011], but it seems that viral RNA can be directly translated after membrane fusion [Lindenbach *et al.,* 2007].

The membrane protein M is the second of the two flavivirus surface proteins. In immature virions it exists as a precursor form prM. After the synthesis and signal peptidase cleavages (Figure 3B) the prM and E proteins fold and form heterodimers at the ER [Lorenz *et al.,* 2002], and interact with the partly assembled nucleocapsids to form immature virions [Li *et al.,* 2008]. During virus maturation, prM is cleaved to mature protein M and an N-terminal “pr” segment.

The cleavage takes place in the trans-Golgi network by the cellular protease furin [Stadler *et al.,* 1997] shortly before the budding of mature virions from the cell. The immature prM is essentially a chaperone: it protects the E protein from undergoing conformational changes induced by the acidic environment in the cell during exocytosis; the mature form of M allows these essential changes when the virus infects a new cell [Heinz and Allison, 2003; Li *et al.,* 2008].

In addition to the three structural proteins C, (pr)M and E, the flavivirus genome codes for at least seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Figure 3). The NS proteins include proteins essential for flavivirus life cycle, e.g. in polyprotein processing, replication, or assembly. NS1 has a role in flavivirus replication, is found both inside and on the surfaces of the infected cells, and secreted from them [Lindenbach and Rice, 2003; Youn *et al.,* 2010]. E.g. in DENV infections, detection of NS1 antigen from serum can be utilized in diagnostics (e.g. [Huhtamo *et al.,* 2010]). NS3 is a multifunctional protein including serine protease, helicase, and RNA triphosphatase activities [Lindenbach and Rice, 2003]. NS5 is the largest and most conserved of the flavivirus proteins [Villordo and Gamarnik, 2009]. Its N-terminal region has a methyltransferase domain, needed for cap (m^7GpppAmp) formation at the 5´-end of the genome, and at the C-terminus, there is the RNA-dependent RNA polymerase function domain [Villordo and Gamarnik, 2009].

There are non-coding regions (NCRs), also known as untranslated regions flanking both ends of the flavivirus RNA coding region. They include important sequences regulating genome replication, translation, and packaging. The 5´-NCR is 90-130 nt at the 5´-end of the RNA [Villordo and Gamarnik, 2009] after which the translation begins at the first methionine [Chambers *et al.,* 1990]. It has a type I cap structure (m^7GpppAmp) and includes e.g. genome cyclization and promoter sequences, essential for RNA replication [Villordo and Gamarnik, 2009]. The 3´-NCR is longer than the 5´-NCR, 400-800 nt [Markoff, 2003]. It has sequences complementary to parts of the 5´-NCR and thus, via base pairing between the 5´- and 3´-NCRs, the viral RNA cyclizes and the 3´-NCR replication-initiation site comes close to the 5´-NCR polymerase recognition site, which allows the polymerase to start RNA minus strand synthesis [Villordo and Gamarnik, 2009]. At later phases after infection, the replication is switched to produce progeny positive strands [Markoff, 2003].

Virus-like particles

The surface proteins prM and E acquire their mature conformations in the ER [Lorenz *et al.,* 2002] and form the envelope of the virus. They are also capable of forming subviral particles or virus-like particles (VLPs) that consist of cell-derived lipid membrane and the viral surface proteins but lack the nucleocapsid. These particles are formed normally during flavivirus infections [Russell *et al.,* 1980] and can be produced in recombinant expression systems [Allison *et al.,* 1995;

Schalich *et al.,* 1996; Heinz and Allison, 2001]. VLPs are smaller than virions. Their diameter is about 30 nm compared to 50 nm of virions, and the buoyant density is about 1.14 g/cm^3 while for whole virions it is 1.19 g/cm^3 [Schalich *et al.,* 1996]. VLPs resemble real virions in their antigenic properties, and thus can be applied in diagnostics and vaccine development.

Ticks as vectors for diseases

Ticks (superorder *Parasitiformes,* suborder *Ixodida*) are hematophageous ectoparasites that feed on warm-blooded animals and are found throughout the world. Like other arachnids (e.g. mites, spiders, harvestmen and scorpions) they are eight-legged arthropods which do not have the compound eyes typical of insects. There are about 900 species of ticks divided into three families: *Argasidae* (argasid or soft-bodied), *Ixodidae* (ixodid or hard-bodied), and *Nuttalliellidae* [Guglielmone *et al.,* 2010] (Figure 4). The last category contains only one species *Nuttaliella namaqua*. According to Guglielmone and others (2010) *Argasidiae* includes six genera and 193 species and *Ixodidae* 14 genera (including *Ixodes, Hyalomma, Dermacentor,* and *Rhipicephalus)* and 702 species, although there are some discrepancies. For example some authors, but not all, consider *Ixodes sachaliensis* and *I. persulcatus* different species [Guglielmone *et al.,* 2010].

Ticks act as vectors for many human and animal pathogens (Tables 1 and 2). A comprehensive table listing tick-borne pathogens and their tick vector species has been published by de la Fuente and others [de la Fuente *et al.,* 2008].

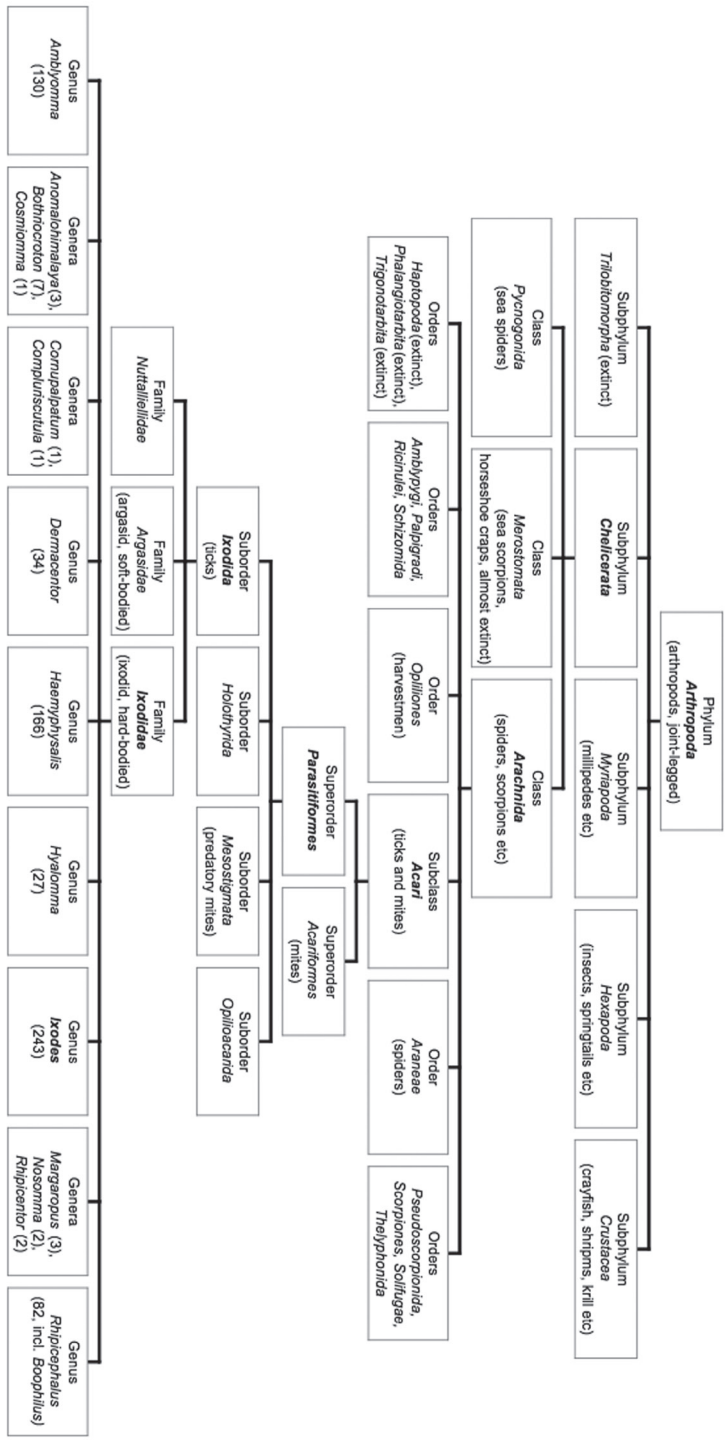

Figure 4. Taxonomic classification of the genus *Ixodes*. The number of recognized species in each genus of the family *Ixodidae* is indicated in parentheses [Guglielmone *et al.,* 2010; ITIS, 2011].

Table 2. Some medically or veterinary important tick-borne pathogens.

Pathogen	Disease	Tick species	Geographical distribution
Babesia bovis	Cattle babesiosis	*Boophilus* spp.	Africa, America, Asia, Australia
B. divergens	Cattle babesiosis; sporadically disease in humans	*Ixodes* spp.	Europe
B. microti	Human babesiosis	*I. scapularis*	USA, Canada
Rickettsia ricketsii	Rocky Mountain spotted fever	*Dermacentor andersoni, D. variabilis, Amblyomma cajennense, A. aureolatum, Rhipicephalus sanguineus*	Americas
R. conorii conorii	Mediterranean spotted fever	*R. sanguineus*	Europe, Africa, Asia
R. sibirica sibirica	Siberian or North Asian tick typhus	*D. nuttalli, D. marginatum, D. sivlarum, D. sinicus, Haemaphysalis concinna*	Asia
R. africae	African tick-bite fever	*Amblyomma hebraeum, A. variegatum*	Africa, Reunion Island, West Indies
R. helvetica	pathogenicity in humans suspected	*I. ricinus*	Europe
Ehrlichia chaffeensis	Human monocytic ehrlichiosis	*A. americanum, D. variabilis*	USA
E. ewingii	Canine granulocytic ehrlichiosis, human ehrlichiosis	*A. americanum*	USA
E. ruminantium	Heartwater	*Amblyomma* spp.	Africa, Caribbean
E. canis	Canine ehrlichiosis	*Rhipicephalus sanguineus*	Southern USA, Southern Europe, Africa, Middle East, Eastern Asia
Anaplasma phagocytophilum	Human granulocytic anaplasmosis	*I. scapularis, I. ricinus, I. pacificus, I. hexagonus*	USA, Europe
A. marginale, A. centrale	Bovine anaplasmosis	Various	Worldwide
Francisella tularensis	tularemia	Various	Eurasia, Nearctic
Coxiella burnetii	Q fever	Various	Worldwide
Borrelia burgdorferi sensu stricto	Lyme borreliosis, mainly arhritis	*I. pacificus, I. persulcatus, I. ricinus, I. scapularis*	USA, Canada
B. garinii	Lyme borreliosis, mainly neuroborreliosis	*I. persulcatus, I. ricinus*	Eurasia, Northern Africa
B. afzelii	Lyme borreliosis, mainly skin symptoms	*I. persulcatus, I. ricinus*	Eurasia, Northern Africa
B. spielmanii	Lyme borreliosis	*I. ricinus*	Europe
B. japonica	Lyme borreliosis	*I. ovatus*	Japan
B. duttonii	Old world tick-borne relapsing fever	*Ornithodoros moubata*	Africa
Theileria mutans	Bening African theileriosis of cattle	*Amblyomma hebraeum, A. lepidum, A. variegatum, A. cohaerens, A. gemma*	Africa
African swine fever virus (genus *Asfivirus,* family *Asfarviridae)*	African swine fever	*Ornithodoros moubata, O. erracticus, O. turcata, O. coriaceus, O. puertiricensis*	Africa, Southern Europe, Caribbean
CCHFV (genus *Nairovirus,* family *Bunyaviridae)*	Crimean-Congo hemorrhagic fever	*Hyalomma marginatum, Hy. anatolicum, Hy. truncatum, A. variegatum, Haemaphysalis punctata, I. ricinus, Dermacentor* spp., *Rhipicephalus* spp.	Africa, Asia, Europe
SFTSV (genus *Phlebovirus,* family *Bunyaviridae)*	Severe fever with thrombocytopenia syndrome	*Haemaphysalis longicornis*	Eastern China
Uukuniemi virus (genus *Phlebovirus)*	Apparently apathogenic	*I. ricinus*	Eurasia
CTFV (genus *Coltivirus,* family *Reoviridae)*	Colorado tick fever	*D. andersonii, D. occidentalis, D. albipictus*	Nearctic
Eyach virus (genus *Coltivirus)*	Encephalitis	*I. ricinus, I. ventalloi*	Central Europe

Pathogen	Disease	Tick species	Geographical distribution
Thogoto virus (genus *Thogotovirus,* family *Orthomyxoviridae)*	Optic neuritis, meningitis	*A. gemma, A. lepidum, Rhipicephalus* spp.	Africa, Europe
OHFV	Omsk hemorrhagic fever	*D. reticulatus*	Western Siberia
LIV	"Louping ill" disease in sheep	*I. ricinus*	British Isles
Powassan virus	Powassan encephalitis	*I. cookie, H. longicornis, I. persulcatus*	USA, Canada, Eastern Russia
Kyasanur Forest virus	Kyasanur Forest disease	*Haemaphysalis* spp.	India
TBEV-Eur	TBE	*I. ricinus*	Europe
TBEV-Sib, TBEV-FE	TBE	*I. persulcatus*	Eurasia

Data compiled from [Charrel *et al.,* 2004; de la Fuente *et al.,* 2008; Jongejan and Uilenberg, 2004], except for SFTSV (severe fever with thrombocytopenia syndrome virus) which has recently been associated with *H. longicornis* ticks in China [Yu *et al.,* 2011], Uukuniemi virus, which is apparently apathogenic but included because prevalent in Finland [Saikku and Brummer-Korvenkontio, 1975], and Thogoto virus [Calisher *et al.,* 1987; Sang *et al.,* 2006]. See also Table 1.

Ticks have three developmental stages: larvae hatch from the eggs and need a blood meal to develop to nymphs, which need a blood meal to develop to adult male or female, which again need blood for reproduction. Nymphs and adults have eight legs but larvae have only six. Ixodid ticks feed once per developmental stage but argasid nymphs and adults take several small blood meals [Anderson and Magnarelli, 2008; Francischetti *et al.,* 2009]. The duration of the developmental cycle varies among tick species and also spatially from less than a year in the tropics to several years in the northern regions. E.g. for *I. persulcatus* in Northern Europe it lasts for 3-5 years [Balashov, 1972].

Tick feeding lasts for 20-70 minutes for the argasid and several days or even weeks for ixodid ticks [Anderson and Magnarelli, 2008]. Sucking blood for days is possible due to several bioactive compounds present in tick saliva, including anticoagulants, platelet aggregation inhibitors, anti-inflammatory substances, and inhibitors of the alternative pathway of complement activation [Wikel, 1996; Steen *et al.,* 2006; Hovius *et al.,* 2008].

When feeding, ixodid ticks concentrate the blood meal and excrete excess water and ions via their salivary glands back to the host [Süss, 2003; Anderson and Magnarelli, 2008; Francischetti *et al.,* 2009]. Each ingestion-salivation cycle lasts for 5-20 min [Francischetti *et al.,* 2009], thus it happens tens or hundreds times during one meal.

Ticks feed at least three times during their life and many species (but not all) on separate warm-blooded hosts. They salivate the contents of their body – possibly including pathogens – to the host during feeding, and stay attached to the host for long times thus can be spread by them even for long distances. These properties make them good vectors for pathogen transmission.

Figure 5. *I. ricinus* larva, nymph, adult male, adult female, and an engorged female. The nail is about 5 cm. Unengorged adult female is approximately 5 mm long. Photo courtesy of Gunnar Hasle [Hasle, 2011], with permission.

Ecology of TBEV

The distribution of the vector for TBEV-Eur, *I. ricinus* (Figure 5), covers the British Islands, continental Europe up to approximately 65°N, Turkey, Caucasus and parts of Northern Africa [Süss, 2003; Lindquist and Vapalahti, 2008; Heyman *et al.,* 2010]. The vector for TBEV-Sib and TBEV-FE, *I. persulcatus,* has its distribution range from western Russia and the Baltic countries through Russia to Japan (Figure 6) [Filippova, 1977; Ecker *et al.,* 1999; Korenberg and Kovalevskii, 1999; Heyman *et al.,* 2010; Süss, 2011] and unexpectedly also at the western coastline of Finland [Jääskeläinen *et al.,* 2006, 2010, 2011]. TBEV, however, is only endemic focally within its tick vectors' distribution areas. This is different from e.g. borreliosis, which is found more or less everywhere where its vector tick species are distributed [Süss, 2003; Lindquist and Vapalahti, 2008].

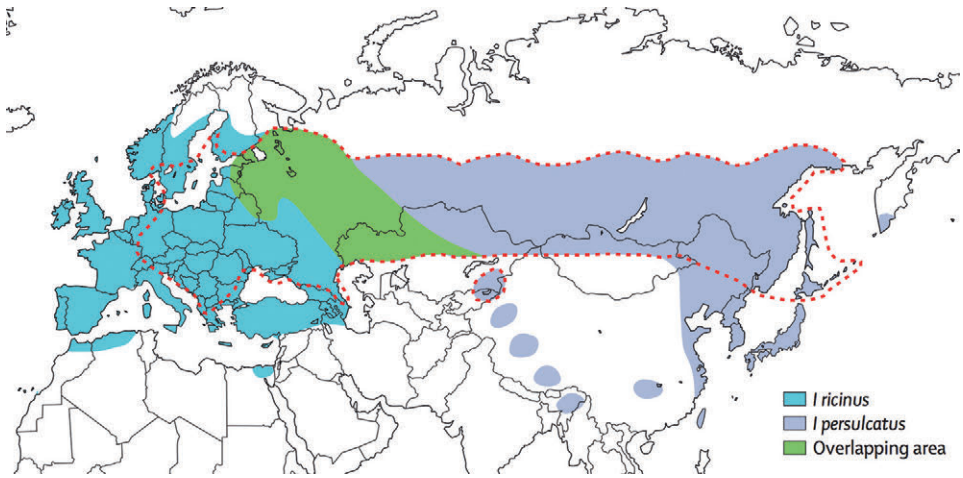

Figure 6. *I. ricinus, I. persulcatus*, and TBEV distribution areas. TBE is endemic focally within the red dotted area. From [Lindquist and Vapalahti, 2008], with permission of the copyright holder.

TBEV circulates in nature between tick vectors and small mammals [Süss, 2003; Bespyatova *et al.,* 2006; Rizzoli *et al.,* 2009; Dobler, 2010]. Both act as reservoir hosts [Süss, 2003]. Once a tick becomes infected with TBEV, it remains infected for the rest of its life including metamorphoses to the next stages [Gritsun *et al.,* 2003b; Süss, 2003; Gubler *et al.,* 2007]. This is called transstadial transmission. Small mammals provide a platform for TBEV circulation and thus act as amplifying reservoir hosts [Süss, 2003].

To be maintained in a given focus in nature, TBEV should be transmitted from an older tick generation to a younger one. Although apparently any developmental stage can infect and become infected and even transovarial transmission has been shown [Řeháček, 1962; Danielová *et al.,* 2010], in practice to effectively circulate in nature, TBEV has to be transmitted from nymphs to larvae as they are far more numerous than adults [Süss, 2003] (Figure 7).

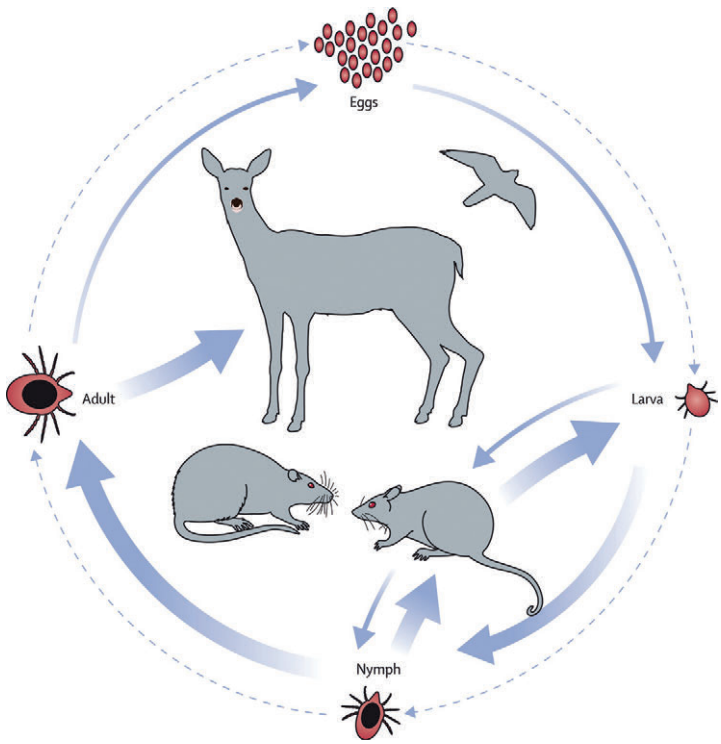

Figure 7. TBEV transmission cycle between *I. ricinus* generations. Dashed line indicates the development of *Ixodes* ticks. Thickness of the solid lines indicates the efficacy of TBEV transmission. The majority of virus transmission happens from infected nymph to uninfected larvae while they cofeed on transmission-competent small mammals. Transovarial transmission is only of minor importance for TBEV circulation [Labuda and Randolph, 1999]. Immature stages (larvae and nymphs) mostly feed on small mammals while adult *I. ricinus* prefer larger animals. Adapted from [Lindquist and Vapalahti, 2008], with permission of the copyright holder.

The transmission cycle of TBEV is fragile. The TBEV-carrying nymphs have to be feeding simultaneously on the same animal (preferably *Apodemus* [Labuda and Randolph, 1999; Briggs *et al.,* 2011], *Myodes,* or *Microtus* [Gubler *et al.,* 2007]) with larvae [Sumilo *et al.,* 2007]. This cofeeding allows the virus to transmit from the nymphs to next generation via the host [Randolph *et al.,* 1996; Korenberg and Kovalevskii, 1999; Randolph *et al.,* 2000]. Actually, despite the anti-inflammatory chemicals in tick saliva, in TBEV-transmission-competent rodents, tick bite still induces inflammation that attracts neutrophils and monocyte/macrophages [Labuda *et al.,* 1996] and TBEV can actually be transmitted between ticks even on an immune animal by these migrating cells [Labuda *et al.,* 1997].

Several factors have to coincide to allow the cofeeding of larvae and nymphs in nature. First of all, the ecological circumstances have to be such that there are enough ticks and rodents. Secondly, the microclimate has to favor the simultaneous activity of different tick developmental stages. In the spring, the

weather has to warm quickly enough from less than +7°C, which is the temperature limit for *I. ricinus* nymphal activity [Knap *et al.,* 2009], to about +10°C, the temperature where also *I. ricinus* larvae are active [Randolph *et al.,* 2000]. *I. persulcatus* temperature limits are unknown [Sumilo *et al.,* 2007]. Moisture is another important factor of microclimate. When the relative humidity is too low, ticks may need to stop questing and instead rehydrate near the soil; on the other hand, in high relative humidity, nymphs may quest higher on the grass and thus attach more likely to bigger, i.e. transmission-incompetent, animals while larvae still prefer questing close to the ground and attach mostly to small mammals [Burri *et al.,* 2011].

After the ticks have fed, the summer has to last long enough for the larvae to mold to nymphs, and at the same time, for the adult females to have their blood meal, mate, and lay eggs which will hatch. Then in the autumn, the cooling has to be rapid so that the newly molded nymphs and the newborn larvae will not have time to quest but will begin to hibernate. Now they should be ready to quest during the following spring at the same time and cofeed to allow TBEV circulation [Randolph *et al.,* 2000]. This can only happen in certain microclimatic conditions, and together with other ecological factors, explains why TBEV is merely patchy endemic within the tick vector area [Randolph *et al.,* 2000; Sumilo *et al.,* 2007]. When the climate changes, the endemic foci may change [Randolph and Rogers, 2000].

However, one should bear in mind that these studies have been done with *I. ricinus* and TBEV-Eur, and it is possible that the other two subtypes, i.e. TBEV-Sib and TBEV-FE, and *I. persulcatus* ticks are not as sensitive to microclimate or that the parameters determining transmission may be different. At least it seems that in Russia, where the latter predominate, TBEV is more uniformly found in areas inhabited by *I. persulcatus*. TBEV-Sib and TBEV-FE RNAs have been detected in offspring of infected male laboratory mice and female wild red voles, respectively [Gerlinskaya *et al.,* 1997; Bakhvalova *et al.,* 2009; Moshkin *et al.,* 2009]. Furthermore, TBEV RNA has recently been detected in wild rodents in winter thus outside the tick-feeding season [Bakhvalova *et al.,* 2006; Tonteri *et al.,* 2011], and infectious TBEV was detected in experimentally infected *Microtus arvalis* three months after infection [Achazi *et al.,* 2011]. The significance of these findings to virus circulation in nature remains to be determined.

In addition to microclimate, also ecological factors such as the density of rodents suitable for TBEV transmission contribute to TBEV ecology. Large amount of rodents leads to a smaller probability of several ticks feeding on the same rodent and thus smaller cofeeding probability, even if the climate would allow it, but too low rodent density will not allow enough ticks to get infected to support TBEV circulation either [Heyman *et al.,* 2010]. Large animals (deer, cow, human etc) do not produce a viremia high enough to infect new ticks [Heyman *et al.,* 2010] or do not usually carry enough ticks close to each other to support the non-viremic cofeeding transmission described for rodents [Labuda *et al.,* 1993; Charrel *et al.,* 2004]. Ungulates still play an important role in maintaining

TBEV-endemic areas indirectly by providing blood meals for adult tick stages for survival and reproduction [Labuda and Randolph, 1999; Süss, 2003; Charrel *et al.,* 2004; Carpi *et al.,* 2008; Rizzoli *et al.,* 2009; Heyman *et al.,* 2010]. The role of birds in the ecology of TBEV is not clear but they may spread the TBEV strains by spreading infected ticks [Jaenson *et al.,* 1994; Süss, 2003; Waldenström *et al.,* 2007].

Epidemiology

TBE is endemic in many European countries, especially in central and eastern Europe, and in Russia. No autochtonous cases have been diagnosed in westernmost Europe including UK, Ireland, the Netherlands, Belgium, Spain or Portugal [Süss, 2011], although close relatives louping ill virus (LIV) and Spanish sheep encephalitis virus (SSEV) are endemic in some of these western European countries. During 1990-2009 an average of about 8500 human cases were registered per year, of which 2800 per year in Europe excluding Russia [Süss, 2011] (Table 3, Figure 8). In Russia, about 58 million people live in the TBE-endemic area which extends almost through the whole country [Süss, 2008]. In addition, TBE is endemic at least in parts of China [Lu *et al.,* 2008], South Korea [Kim *et al.,* 2008, 2009], Kyrgystan [Briggs *et al.,* 2011], Japan, and Mongolia [Süss, 2008].

Table 3. TBE cases in some European countries 1990-2009.

	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Austria	89	128	84	102	178	109	128	99	62	41	60	52	60	82	54	100	84	45	86	79
Belarus			2	20	50	66	97	67	78	26	23	61	18	25						
Croatia	23	60	27	76	87	59	57	25	24	26	18	27	30	36	38	28	20	12	20	
Czech R.	193	356	338	629	613	744	571	415	422	490	719	411	647	606	500	642	1029	542	630	816
Denmark									1	4	3	1	1	4	8	4		2	1	1
Estonia	37	68	163	166	177	175	177	404	387	185	272	215	90	237	182	164	171	140	90	179
Finland	5	8	12	20	17	24	10	20	17	12	41	33	38	16	31	17	18	20	23	26
France	2	1	2	5	4	6	1	1	2	5	0	0	2	6	7	0	6	7	10	
Germany		44	142	118	306	226	114	211	148	115	133	253	226	278	274	431	546	238	285	313
Hungary	222	288	206	329	258	234	224	99	84	51	45	76	80	114	89	52	56	62	70	64
Italy	0	0	2	2	8	6	8	8	11	5	15	19	6	14	23	22	14	4	34	32
Latvia	122	227	287	791	1366	1341	716	874	1029	350	544	303	153	365	251	142	170	171	181	328
Lithuania	9	14	17	198	284	426	309	645	548	171	419	298	168	763	425	242	462	234	220	617
Norway									1	1	1	0	2	1	2	3	3	13	9	8
Poland	8	4	8	249	181	267	257	201	209	101	170	205	126	339	262	174	316	233	202	335
Russia	5486	5225	6301	7893	5596	5982	9548	6539	6987	9955	5931	6339	5150	4770	4235	4551	3510	3098	2817	3721
Slovak R.	14	24	16	51	60	89	101	76	54	57	92	76	62	74	70	28	91	46	77	71
Slovenia	235	245	210	194	762	260	406	274	136	150	190	260	262	282	204	297	373	199	246	307
Sweden	54	75	83	51	116	68	44	76	64	53	133	128	105	105	160	130	163	190	224	211
Switzerland	26	27	66	44	97	60	62	123	68	112	91	107	53	116	138	206	259	113	127	118
Total	**6525**	**6794**	**7966**	**10938**	**10160**	**10142**	**12830**	**10157**	**10332**	**11910**	**8900**	**8864**	**7279**	**8233**	**6953**	**7233**	**7291**	**5369**	**5352**	**7226**

Data from [Süss, 2011], except for Belarus: [Lindquist and Vapalahti, 2008], and for Finland 1990-1998: Olli Vapalahti, HUSLAB (Diagnostic Laboratory of the Hospital District of Helsinki and Uusimaa) and University of Helsinki, personal communication.

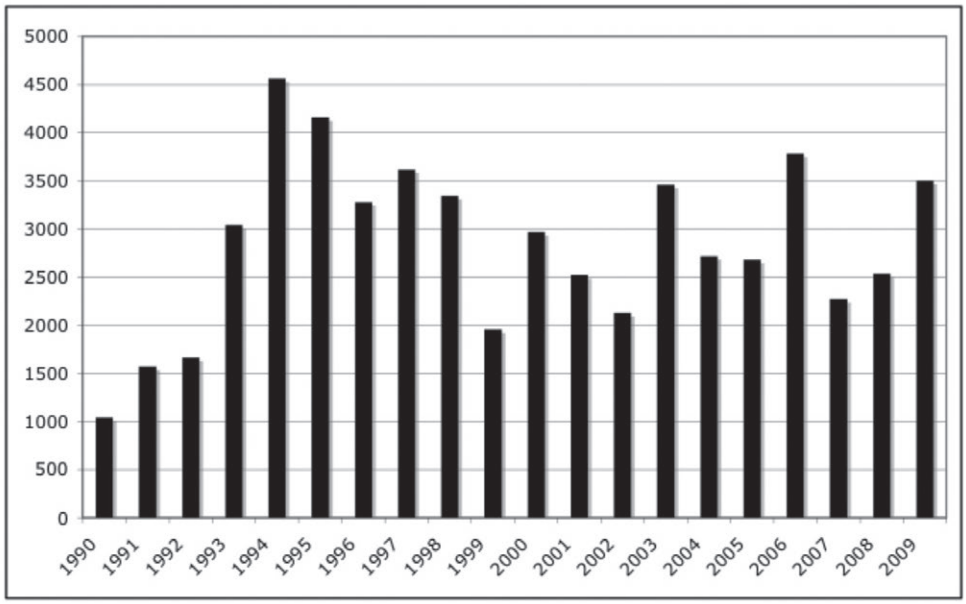

Figure 8. TBE cases in Europe excluding Russia 1990-2009 [Lindquist and Vapalahti, 2008; Süss, 2011].

The epidemiology of TBE is and has been changing. From 1970s an average increase of 400% in TBE incidence in TBE-endemic countries in Europe, except for Austria, has been observed [Süss *et al.,* 2008; Süss, 2011]. In particular in the 1990s in many central and eastern European countries a dramatic upsurge of TBE cases was observed [Sumilo *et al.,* 2007; Randolph, 2010] (Table 3). The increase of TBE incidence correlates with observed climate change [Daniel *et al.,* 2009]. Furthermore, TBEV has appeared in new areas, including Denmark [Fomsgaard *et al.,* 2009] and northern Finland [Jääskeläinen *et al.,* 2011] and also seems to have disappeared from foci previously endemic [Klaus *et al.,* 2009]. In mountainous regions in Central Europe ticks are nowadays found at higher altitudes than before: in Czech Republic at Šumava Mountains, in a survey performed in 1957 *I. ricinus* was found maximally at 800 m, and in 2001-02 at 1100 m above sea level [Daniel *et al.,* 2003]. Consequently, also TBE human cases have been found higher in the mountains [Holzmann *et al.,* 2009; Lukan *et al.,* 2010].

Establishment of TBE in new areas is apparently partly due to observed global climate change (e.g. [Lukan *et al.,* 2010]), and new endemic areas have been predicted according to global warming models [Randolph and Rogers, 2000]. Occurrence of many vector-borne diseases is dependent on climatic and microclimatic factors due to the sensitivity of the arthropod vectors to climate. The same applies for TBE. Temperature and humidity influence on 1) the abundance and dynamics of ticks [Perret *et al.,* 2004], e.g. *I. ricinus* needs ≥7°C air temperature [Öhman, 1961; Knap *et al.,* 2009] and >85% humidity [Süss

et al., 2008] for questing; 2) vegetation and fauna, including ticks' vertebrate host densities and species diversity; and 3) TBEV-enzootic cycle which requires nymphs and larvae to be active simultaneously [Randolph *et al.,* 2000]. Temporal weather conditions further influence on human behavior and outdoor activities. However, changes in the epidemiology of TBE cannot be explained entirely by the impact of climate change. Actually, a true climate change has been observed in TBE-endemic countries during the same time as the increase in TBE cases [Sumilo *et al.,* 2007; Daniel *et al.,* 2009], but in some cases the increase of temperature happened actually shortly after the increase in TBE cases [Randolph, 2004]. Vector-borne disease systems in general are complex networks of pathogen-vector-host relationships, and at least for TBE, human actions play an important role.

A spike in TBE incidence was seen in several European countries in 2006, apparently due to exceptionally dry and warm summer and autumn, which did not affect the activity of ticks but instead added human visits to forests and parks and thus human contacts to ticks [Daniel *et al.,* 2008; Randolph *et al.,* 2008; Godfrey and Randolph, 2011]. On the other hand, increased TBE incidence in Latvia, Lithuania, and Poland in 2009 could not be linked to weather variables. Instead, Godfrey and Randolph saw a correlation between spikes in TBE incidence and increased unemployment and poverty, probably due to decreased uptake of costly vaccines and increased mushroom and fruit picking for sales [Godfrey and Randolph, 2011].

A debate is going on about the influence of politics, namely collapse of the Soviet Union, on TBE epidemiology. In the early 1990s the incidence of TBE increased from 0.5-7 to 11-54 new cases per 100 000 population per year in Estonia, Latvia, and Lithuania [Sumilo *et al.,* 2007]. This coincides with the independence from Soviet rule. The dramatic political changes that occurred in the Baltic countries (and other central and eastern European countries) following the independence resulted in changes in human behavior and the risk of humans to be exposed to TBEV infection [Randolph and EDEN-TBD sub-project team, 2010]. These changes observed in human action include decline of industry, thus reduction of pollution [Sumilo *et al.,* 2007], and in Estonia, reduction of pesticide usage since 1992 [Sumilo *et al.,* 2008]. Agricultural reorganization and shift of land usage from farmed to wooded land [Sumilo *et al.,* 2007] together with changes in land ownership [Vanwambeke *et al.,* 2010] have an impact on tick and their host abundance and on human visits to forested areas [Randolph and EDEN-TBD sub-project team, 2010]. Furthermore, fewer people were routinely vaccinated against TBE than in the former Soviet Union [Gritsun *et al.,* 2003b]. Increased unemployment and adjustment to market economy led to increased poverty and also increased wealth among other segments of the population and thus changes in individuals lifestyle, including leisure outdoor activities [Randolph, 2008]. However, contrary to the data presented by Sarah Randolph and others, some authors did *not* find any correlation between socio-economic factors possibly caused by the changed political situation in central and eastern Europe [Kříž *et*

al., 2004] but prefer the observed climate change as the only explanation for the increased TBE incidence in Europe [Daniel *et al.,* 2009].

TBE in Finland

Finland is situated at the northernmost edge of the TBE-endemic area in Europe and here TBE is merely focally endemic. An aseptic encephalitis has been known in Kumlinge, an island and a parish in the Åland Islands, since the 1940s [Oker-Blom, 1956; Wahlberg *et al.,* 1989] - hence the disease is also known as Kumlinge disease. According to a legend, a tick-borne encephalitis-like disease would have been known in the Åland Islands already in the 18th century (Kunz and Heinz, 2003). Apparently this however is a misunderstanding, probably due to a doctoral thesis "Om skärgårds febren omkring Åbo" (Concerning archipelago fever in Turku region), published in 1781, which describes malaria, not tick-borne encephalitis (Brummer-Korvenkontio, 2007).

Since the 1960s the TBE-endemic foci in Finland have included the Åland Islands and the archipelago of Turku, archipelago of Kokkola, and Lappeenranta region in the southeast [Tuomi and Brummer-Korvenkontio, 1965]. These were determined by studying cow serum samples from all over Finland for antibodies against TBEV [Tuomi and Brummer-Korvenkontio, 1965] (Figure 9A). These areas have remained the same throughout decades [Jääskeläinen *et al.,* 2006, 2010]. In addition, Isosaari island in the archipelago of Helsinki was found to be endemic for TBE in the 1990s [Han *et al.,* 2001]. Sporadic human cases have appeared since in the western coast in Närpiö and in eastern Finland in Varkaus [Jääskeläinen *et al.,* 2010], in the Kuopio region [National Institute for Health and Welfare, 2011], and in Kotka (Teija Korhonen, National Institute for Heath and Welfare, personal communication). 2008 and 2009 a few human cases were traced to Simo in southern Lapland [Jääskeläinen *et al.,* 2011] (Figure 9B).

Tick distribution in the country has not been properly studied since the 1950s [Öhman, 1961]. At that time only observations of *I. ricinus* south from Kokkola-Joensuu axis were made (Figure 9A).

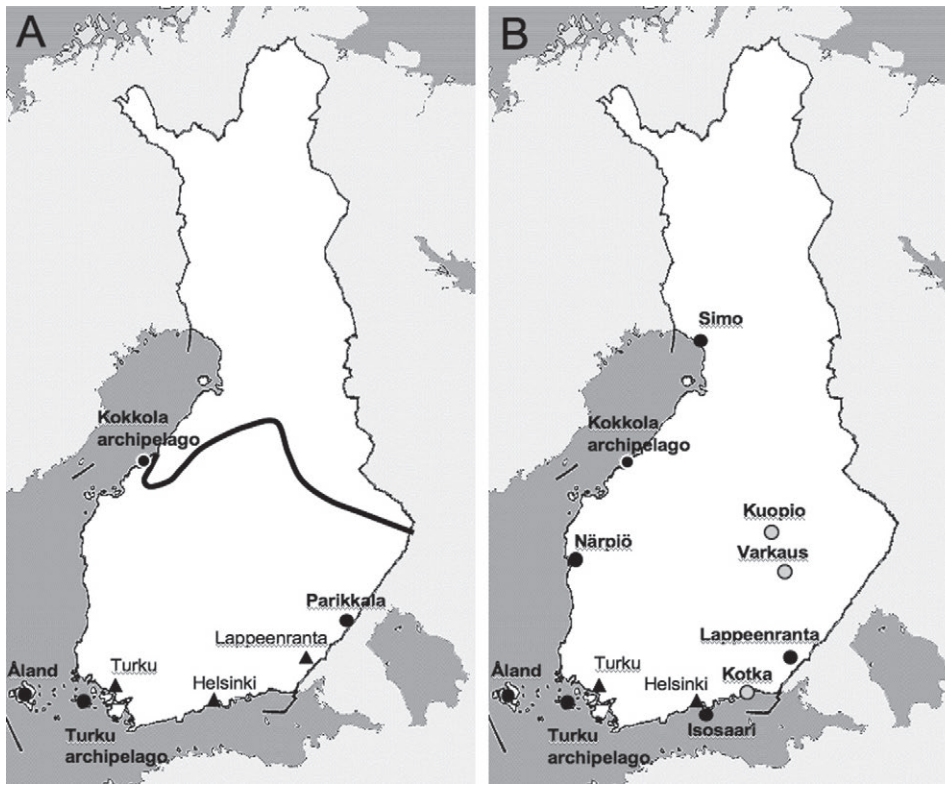

Figure 9. A. The line indicates *I. ricinus* distribution in Finland in 1950s [Öhman, 1961] and the black circles the geographical ditribution of TBEV antibodies in cattle sera in the 1960s [Tuomi and Brummer-Korvenkontio, 1965]. TBEV strain Joutseno was isolated from *I. ricinus* pool from Lappeenranta region in 1960 [Brummer-Korvenkontio *et al.,* 1973]. **B.** The black circles indicate the TBE-endemic areas in Finland now and the grey circles show where sporadic human TBE cases have been registered, one case in Varkaus, 2008, and two in Kuopio region 2009-10 and in Kotka 2010 [National Institute for Health and Welfare, 2011; Teija Korhonen, National Institute for Health and Welfare, personal communication].

The incidence of TBE in the Åland Islands has been the highest in Finland, and high even in international scale: in 2001-2009 the incidence in Åland was 11-93 per 100 000 inhabitants per year [National Institute for Health and Welfare, 2011] (Figure 10). Traditionally, approximately two thirds of Finnish acute TBE cases have been from Åland, where the population is only about 28 000. Lately however, possibly partly due to the mass vaccination campaign supported by the government that started in Åland in 2006, the relationship has changed so that most of the new cases come from mainland Finland, where the number of cases has increased. During the last ten years, 16-41 new cases were yearly diagnosed [National Institute for Health and Welfare, 2011].

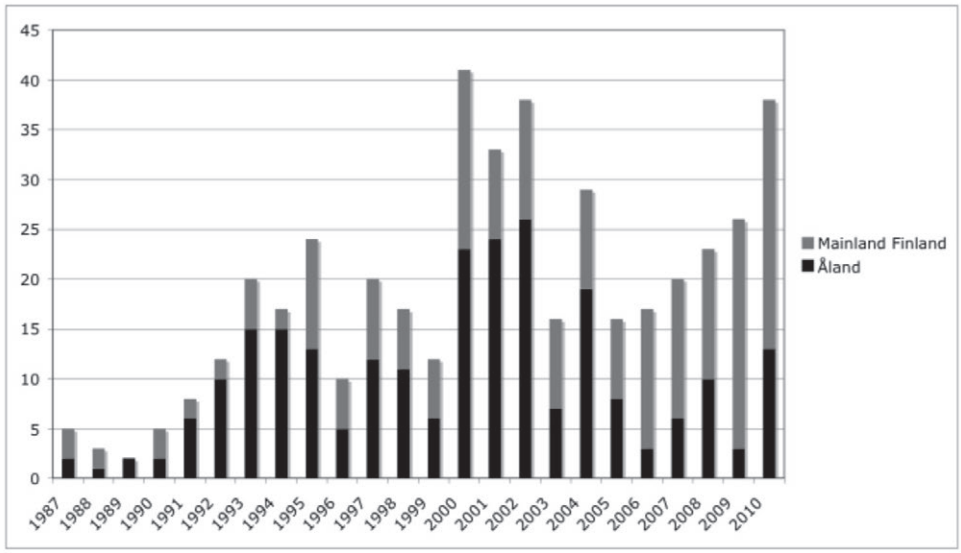

Figure 10. TBE cases in Finland 1987-2010. 1987-1998: data from Olli Vapalahti, HUSLAB (Diagnostic Laboratory of the Hospital District of Helsinki and Uusimaa) and University of Helsinki, personal communication; 1999-2009: data from the Infectious Diseases Registry [National Institute for Health and Welfare, 2011]; 2010: data from Teija Korhonen, National Institute for Heath and Welfare, personal communication.

Clinical picture of TBE

TBEV circulates in nature mainly between ticks and small mammals while humans are only accidental hosts. Humans can get tick-borne encephalitis from an infected tick bite, which however is not always noticed [Günther *et al.,* 1997; Kaiser, 1999; Lindquist and Vapalahti, 2008; Bogovic *et al.,* 2010]. TBEV survives in the alimentary tract and people can become infected also from consumption of unpasteurized milk from an infected goat or cow [Kerbo *et al.,* 2005; Holzmann *et al.,* 2009; Balogh *et al.,* 2010]. Occasional laboratory infections [Wahlberg *et al.,* 1989; Avšič-Županc, *et al.,* 1995] or infections from blood transfusions [Wahlberg *et al.,* 1989] have also been reported.

In humans the clinical picture of TBEV infection varies. Approximately two thirds of infections are asymptomatic or subclinical [Kaiser, 2008]. In those who get symptoms, TBE is often biphasic, at least in areas where the TBEV-Eur subtype dominates [Oker-Blom, 1965; Kaiser, 1999; Haglund and Günther, 2003; Wahlberg *et al.,* 2006; Lindquist and Vapalahti, 2008]. The first phase, which begins approximately 1-3 weeks after the tick bite (median 8 days, range 4 to 28 days according to Kaiser [1999]; median 22 days, range 4 to 34 days according to Czupryna and others [2010]), is a flu-like febrile illness of a few days [Kaiser, 1999, 2008; Bogovic *et al.,* 2010] after which an asymptomatic period of about a week (median 7 days, range 3 to 12 days according to Kaiser [1999]) takes place before the more severe second phase of acute TBE with central nervous system

manifestations. TBE symptoms are classified as mild (mainly meningeal signs such as fever, headache, nausea, or rigidity of the neck), moderate (encephalitic symptoms, including e.g. monofocal brain symptoms or moderate central nervous system dysfunction, e.g. slightly decreased level of consciousness), and severe (meningoencephalomyelitic symptoms, multifocal brain symptoms or severe dysfunction of the central nervous system) [Günther *et al.,* 1997]. Severe acute TBE symptoms include e.g. decreased consciousness, ataxia and paresis, including respiratory failure, and may demand intensive care [Günther *et al.,* 1997; Kaiser, 1999; Jereb *et al.,* 2002; Lindquist and Vapalahti, 2008; Czupryna *et al.,* 2010]. In studies from Sweden and Lithuania, where the European subtype of TBEV circulates, the mild form was seen in 44-55%, moderate in 34-37% and severe in 8-13% of the patients [Günther *et al.,* 1997; Mickienė *et al.,* 2002]. The severity of TBE increases with age [Kaiser, 1999; Mickienė *et al.,* 2002; Lindquist and Vapalahti, 2008; Czupryna *et al.,* 2010].

About one third of patients have been reported to have long-term sequelae (36% [Haglund *et al.,* 1996]; 40% [Günther *et al.,* 1997]; 27% [Kaiser, 1999]; 31% [Mickienė *et al.,* 2002]; 23% neurological and 44% psychiatric sequelae [Czupryna *et al.,* 2010]). The case fatality rate in Europe is less than 2% [Donoso Mantke *et al.,* 2007b; Lindquist and Vapalahti, 2008]. The TBEV-Sib subtype seems to have a tendency to develop a chronic TBEV infection more often than TBEV-Eur [Gritsun *et al.,* 2003a, 2003b; Charrel *et al.,* 2004; Poponnikova, 2006; Donoso Mantke *et al.,* 2007b; Lindquist and Vapalahti, 2008] and the reported case fatality rates are 2-8% [Gritsun *et al.,* 2003b; Charrel *et al.,* 2004; Lindquist and Vapalahti, 2008; Mansfield *et al.,* 2009]. The TBEV-FE infection is the most severe. Focal forms of TBE, including meningeal symptoms and focal lesions in central nervous system which may manifest e.g. as paresis depending on the localization of the lesions [Chekhonin *et al.,* 2002], are more often associated with TBEV-FE than with the other subtypes [Gritsun *et al.,* 2003b] and high fatality rates after TBEV-FE infection have been reported [Gritsun *et al.,* 2003b; Donoso Mantke *et al.,* 2007b; Lindquist and Vapalahti, 2008; Mansfield *et al.,* 2009]. However, although it seems that the disease caused by the TBEV-Eur may be milder than those caused by the other two subtypes [Bogovic *et al.,* 2010; Heyman *et al.,* 2010], the differences seen in morbidities may partly be due to the other reasons, e.g. possible differences in reporting or hospitalization rates in different countries [Lundkvist *et al.,* 2001; Heinz *et al.,* 2007; Lindquist and Vapalahti, 2008; Heyman *et al.,* 2010].

There is no specific treatment for TBE [Lindquist and Vapalahti, 2008] but a patient is treated according to the symptoms, e.g. anti-inflammatory drugs may be used to reduce the inflammation and intracranial pressure [Czupryna *et al.,* 2010].

Diagnostics

The symptoms of TBE are unspecific and a similar clinical picture may be caused by many infections, so the diagnosis has to be established in a laboratory. For early diagnosis, detection of viral nucleic acid from serum by RT-PCR could, in theory, be used, and actually is in some laboratories [Donoso Mantke *et al.,* 2007a, 2008], but it is of limited usefulness as the patients are viremic only for a very short period of time during the first phase of the illness [Schultze *et al.,* 2007] when the symptoms are typically rather mild. Often patients seek medical help not earlier than at the second phase of the disease when the more severe neurological symptoms appear. At this phase the virus has already been cleared from the blood and isolation of TBEV or detection its nucleic acid or antigen is usually no longer successful [Puchhammer-Stöckl *et al.,* 1995; Holzmann, 2003; Donoso Mantke *et al.,* 2007b]. Consequently, laboratory diagnostics is based on serology [Holzmann, 2003]. IgM and IgG antibodies in serum are found at the beginning of the second phase of the disease [Jereb *et al.,* 2002; Holzmann, 2003], and in the cerebrospinal fluid by the tenth day [Holzmann, 2003]. IgM antibodies can be detected from serum for several months after infection, and IgG antibodies persist forever providing lifelong immunity [Holzmann, 2003]. Due to possible unspesific reactions, a positive IgM finding should be confirmed by demonstration of IgG conversion. Magnetic resonance imaging, X-ray computed tomography, or electroencephalography findings are non-specific and have no value in differential diagnostics [Czupryna *et al.,* 2010].

Commonly used serological tests for TBE diagnostics include:

Enzyme immunoassay (EIA). In an enzyme immunoassay (also known as enzyme-linked immunosorbent assay or ELISA) the antigen-antibody binding is recognized by an enzyme-labeled conjugate, and the enzyme is then offered a substrate to visualize the binding reaction, e.g. by changing color. There are several commercial EIA kits available for TBE IgG and IgM diagnostics [Niedrig *et al.,* 2001] and EIA is one of the most popular choices for TBE diagnostics [Donoso Mantke *et al.,* 2007b, 2008].

Immunofluorescence assay (IFA). In an immunofluorescence assay the presence of antibodies – or antigen – is detected by a conjugate labeled with a fluorescent marker. The cells expressing the antigen are permeabilized and fixed e.g. with acetone onto a microscope slide. Diluted serum sample is incubated on the slide, and antibody-antigen binding can be detected by an anti-immunoglobulin conjugate labeled with a fluorescent marker and viewing with a fluorescence microscope.

Virus neutralization test (NT). Neutralization is based on the ability of specific antibodies to reduce the infectivity of a virus. Neutralizing antibodies are often type-specific and in the case of flaviviruses, they do not cross-react as strongly as antibodies detected by other serological methods. Therefore, NT should be considered for confirmation of a positive TBEV diagnosis in areas where TBE is not known to be endemic [Holzmann, 2003] or where several flavivirus

circulate, e.g. WNV and TBEV in Hungary [Ferenczi *et al.,* 2008]. Neutralization requires live virus and in the case of TBEV and many other flaviviruses has to be performed in a biosafety level 3 laboratory.

In NT a cell culture is infected with live virus suspension that has been incubated with different dilutions of a serum sample. If there are neutralizing antibodies in the serum, the corresponding virus does not infect the cells. Applications of NT include rapid fluorescent focus inhibition test, described for TBEV by Vene and others [1998].

Hemagglutination inhibition assay (HI). Hemagglutination is a phenomenon where a viral protein, also called hemagglutinin for some viruses, interacts with receptors on the surface of red blood cells and cross-links them. TBEV E protein is capable of hemagglutination [Holzmann *et al.,* 1996]. The E protein also induces formation of antibodies that inhibit the hemagglutination ability. In the HI test, the patient serum is preadsorbed e.g. with kaolin and erythrocytes to remove non-specific agglutinating factors. The adsorbed sample is diluted and hemagglutination studied with goose or chick erythrocytes and inactivated virus antigen [Clarke and Casals, 1955, 1958], e.g. TBEV. If the sample has no TBEV antibodies, the red blood cells will hemagglutinate. If antibodies in the sample inhibit the agglutination of the erythrocytes (the erythrocytes form a red pellet at the bottom of the sample well) the patient has antibodies to TBEV, and the titer is the last dilution where the inhibition takes place. HI test cannot routinely distinguish between Ig subclasses (IgG and IgM).

The common cross-reactivity among flaviviruses in serological tests provides a challenge for diagnostics [Allwinn *et al.,* 2002]. Other flavivirus infections (e.g. DENV, WNV, or JEV infections) as well as vaccinations to flavivirus infections (e.g. live attenuated yellow fever vaccine) may cause false-positive results in TBE serology [Niedrig *et al.,* 2001]. Sometimes false positives can be ruled out by the known travel and vaccination history of the patient – within TBE-endemic areas TBE is often the only circulating flavivirus pathogenic to humans. However, in Central Europe and Russia, WNV may in theory be infecting humans in areas overlapping with TBE endemicity. NT should be used to confirm positive serology in ambiguous cases [Donoso Mantke *et al.,* 2008]. A phenomenon called "original antigenic sin" may disturb the flavivirus diagnostics and should be remembered if the patient may have had several flaviviral infections [Halstead *et al.,* 1983; Holzmann *et al.,* 1996]. The antibodies formed against a certain virus may actually be boosted by an infection with a related but not necessarily identical virus. Consequently it may be the first and not the new virus that shows the highest antibody levels in serological tests [Krause, 2006].

Currently there is apparently no test available for serological differentiation of different TBEV subtypes [Donoso Mantke *et al.,* 2007b].

Prevention

Two vaccines against TBE are in the market in the EU, TicoVac® (formerly FSME-Immun) by Baxter, and Encepur® by Novartis Vaccines (formerly Chiron, formerly Behring) [Heinz *et al.,* 2007; Demicheli *et al.,* 2009]. They are essentially similar, based on TBEV-Eur strains Neudörfl (TicoVac) and K23 (Encepur), formaldehyde-inactivated whole virus particles grown in chick embryos, except that TicoVac is stabilized by human albumin and Encepur by sucrose [Heyman *et al.,* 2010]. In addition at least two vaccines are produced and available in Russia, a vaccine manufactured by Chumakov Institute of Poliomyelitis and Viral Encephalitides RAMSci (Moscow, Russia) and EnceVir by Scientific Production Association Microgen (Tomsk, Russia) [Leonova and Pavlenko, 2009], and one in China, produced by Changchun Institute of Biological Products (Changchun, China) [Lu *et al.,* 2008]. These are based on TBEV-FE strains Sofjin, 205, and Senzhang, respectively. Apparently at least the TBEV-Eur based vaccines are protective against all TBEV subtypes [Holzmann *et al.,* 1992; Schmaljohn *et al.,* 1997; Hayasaka *et al.,* 2001; Leonova and Pavlenko, 2009; Heyman *et al.,* 2010]. A three-dose course within a year and boosters after every three to five years are recommended for protection. Vaccination provides good protection, as seen in Austria where the vaccine has been widely used since the 1970s and the vaccination coverage is nowadays 58% (88% has received at least one dose) and the annual TBE cases have been reduced from several hundreds in the early 1980s to about ten in the beginning of 2000s [Heinz *et al.,* 2007]. Nevertheless, vaccine failures even after complete series of vaccine doses have been reported as well [Bender *et al.,* 2004; Andersson *et al.,* 2010; Grgič-Vitek *et al.,* 2010].

One can try to avoid TBE and other tick-borne infections by avoiding tick bites. When moving in forests and meadows, covering legs with long trousers and boots could be considered. Ticks often do not bite immediately when they find a host but may spend hours for searching an attractive site in the skin to pierce; in humans this means areas where skin is thin and soft and has blood vessels close to the surface, e.g. crooks, groins or scalp. Thus it can be recommended to check oneself and others after spending time in tick-infested areas. TBEV apparently transmits within minutes after the tick bite [Lindquist and Vapalahti, 2008], like does another flavivirus Powassan virus [Ebel and Kramer, 2004], but removing an already attached tick can still reduce the risk of contracting borreliosis, even 24 h after the tick bite [Piesman *et al.,* 1987]. When possible, one should consider avoiding consumption of milk products made of unpasteurized milk of goats or cows from TBE-endemic areas.

In some cases it may be wise to try to reduce the tick population from a restricted area such as a garden or a children's playground. Tricks to try include lawn mowing, discouraging rodent activity, and placing children's swings etc apart from the woods and bushes [Stafford, 2004].

Aims of this study

TBEV is classified as a biosafety level 3 pathogen, thus handling it is laborious and requires specific laboratory facilities. One aim was therefore to develop a non-infectious recombinant antigen suitable for TBE IgM detection in a μ-capture format for routine diagnostics of acute TBE.

Not much is known about the molecular epidemiology of TBEV in Finland. Vaccines for TBE are widely sold in Finland and elsewhere in Europe but estimations of the need of vaccination and risk areas are rarely based on investigated information about the actual TBE risk. Specific aims included

- defining the prevalence of TBEV in ticks by studying ticks from the previously known TBE-endemic foci in Finland as well as from sites which recently have appeared as possibly TBE-endemic. Two targets from Russia, the republics of Karelia and Buryatia, were also selected.

- studying the dispersion and distribution of TBEV strains to estimate how the Finnish TBE-endemic foci are formed and maintained. TBEV strains were isolated from ticks, small mammals and human sera to further characterize the TBEV strains circulating in Finnish, Karelian, and Buryatian TBEV foci.

Materials and methods

Materials

Reference virus strains (I, II, III, IV)

To develop a recombinant TBEV-prME antigen (Publication I), we used the Finnish prototype strain Kumlinge A 52, which has been isolated from an *I. ricinus* pool from Kumlinge in the Åland Islands, south-western Finland, in 1959 [Brummer-Korvenkontio *et al.,* 1973], and whose complete open reading frame has recently been sequenced [Uzcátegui *et al.,* unpublished]. The same strain was used as European subtype positive control for RT-PCRs for screening ticks (II, III, IV), wild rodents (IV), and the virus isolation experiments (II, III, IV).

As Siberian subtype positive control for RT-PCRs we used strains Kokkola-79 and Kokkola-118, isolated from *I. persulcatus* pools from Kokkola archipelago, western Finland, in 2004 (Publication II), and as Far-Eastern subtype control, the strain Sofjin-HO, isolated from Russia in 1937 (apparently strain "Sof" isolated from a patient in Russia in 1937 [Silber and Soloviev, 1946] equals Sofjin), and kindly provided by Sirkka Vene, Swedish Institute for Communicable Disease Control.

TBEV-Eur and TBEV-Sib positive control RNAs were isolated from infected mouse brains and TBEV-FE from Vero E6 cells by TriPure® Isolation Reagent (Roche Diagnostics, Espoo, Finland). RNAs were aliquoted as 10^{-3} dilutions in diethylpyrocarbonate-treated H_2O at -70°C to be used and further diluted for RT-PCRs for screening samples for TBEV RNA and for sensitivity comparisons of conventional NS5- [Puchhammer-Stöckl *et al.,* 1995], modified by us (Publication II), and 5´-NCR- [Schrader and Süss, 1999] RT-PCRs, and for real-time RT-PCR [Schwaiger and Cassinotti, 2003], modified by us [Tonteri *et al.,* 2011].

Cell lines (I, II, III, IV)

Cell lines used for recombinant baculovirus expression (Publication I) were insect cell lines Sf9 *(Spodoptera frugiperda,* fall armyworm, ATCC CRL-1711) and High Five™ *(Trichoplusia ni,* cabbage looper, Invitrogen, Carslbad, CA) grown as monolayers in SF-900 medium (Invitrogen) supplemented with 10% fetal calf serum, amphotericin B, glutamine, penicillin and streptomycin at +27°C.

For virus isolation experiments *in vitro* and passaging virus strains we used Vero E6 *(Cercopithecus aethiops,* African green monkey kidney, ATCC CRL-1586) cells. These were grown as monolayers on minimum essential medium supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin at +37°C and 5% CO_2.

Reference sera (I)

To test baculovirus-expressed TBEV-prME proteins' ability to work as an antigen in a μ-capture TBEV IgM EIA test, we used 157 human serum samples sent to the diagnostic laboratory of the Hospital District of Helsinki and Uusimaa, HUSLAB, Helsinki, Finland (formerly Helsinki University Central Hospital HUCH Laboratory Diagnostics) due to suspicion of TBE or dengue. 50 TBEV-IgM positive, two of which were positive for rheumatoid factor, 100 TBEV-IgM negative, and seven DENV-IgM positive sera were used as reference samples. Details are described in Publication I. Later we used additional 418 TBEV-IgM negative and six TBEV-IgM positive sera to further evaluate the μ-capture IgM EIA test developed.

Monoclonal antibodies (I)

The antigenic properties of baculovirus-expressed TBEV E protein were studied by five monoclonal antibodies against TBEV-Eur strain K23 E protein [Niedrig *et al.,* 1994].

Tick panels (II, III, IV)

We chose the tick collection sites based on patient interviews done by the Institute of Health and Welfare or by local medical experts (Tapani Tikkakoski, Kokkola archipelago; Nataliya Subbotina, Russian Karelia; Galina B. Murueva, Buryatia). The tick panels are described in Table 4.

Table 4. Tick panels used for screening TBEV RNA.

Tick panel	Females	Males	Nymphs	Larvae	Total	Pools	Species
Kumlinge 2003 (III)	44	23	387	0	454	45	*I. ric*
Kokkola archipelago 2003	0	1[1]	138[1]	0	139	15	ND[1]
Kokkola archipelago 2004 (II)	570	539	72	0	1181	122	*I. per*
Buryatia 2005 (III)	128	166	2	0	296	NA	*I. per*
Isosaari 2005 (III)	16	26	54	0	96	11	*I. ric*
Lappeenranta 2005 (III)	140	138	14	0	292	29	*I. ric*
Russian Karelia 2006 (III)	110[2]	66[2]	22	0	198	NA	Both[2]
Turku archipelago 2007 (III)	90	82	863	4	1039	315	*I. ric*
Närpiö 2008 (III)	22	14	0	0	36	NA	*I. per*
Varkaus 2009	1	2	7	0	10	4	*I. ric*
Simo 2009 (IV)	43	54	0	0	97	51	*I. per*
Lappeenranta 2010	54	45	2	0	101	11	*I. ric*
Kuopio 2010	18	14	1	0	33	4	*I. ric*
Total	**1236**	**1170**	**1562**	**4**	**3972**		
of which *I. ric*	364	334	1328	4	2030		
and *I. per*	872	835[1]	96[1]	0	1803[1]		

[1]Kokkola 2003: not determined; probably should be included in *I. persulcatus*. [2]Karelia 2006: 193 *I. persulcatus,* 4 *I. ricinus* males, 1 *I. ricinus* female. *I. ric, Ixodes ricinus*. *I. per, Ixodes persulcatus*. ND, not determined. NA, not applicable.

Human sera used in virus isolation experiments (III)

Table 5. Patient serum virus isolation experiments.

Patient	Sample applied for virus isolation trial	Later sample with IgM antibodies	Mice	Cell culture
2000	15 Aug 2000 HI<10, IgM neg	2 Sep 2000 HI>640, IgM pos		X
2001-A	7 Aug 2001 HI<20, IgM neg	4 Sep 2001 HI=160, IgM pos	X	X
2001-B	8 Aug 2001 HI=10, IgM pos	24 Aug 2001 HI=320 IgM pos	X	X
2001-C	12 Aug 2001 HI<20, IgM neg	31 Aug 2001 HI=320, IgM pos	X	X
2001-D	1 Oct 2001 HI<10, IgM neg	11 Oct 2001 HI=160, IgM pos	X	X
2002-A	23 Jun 2002 HI<10, IgM neg	8 Jul 2002 HI=1280, IgM pos	X	X
2002-B	8 Jul 2002 HI<10, IgM neg	15 Jul 2002 HI=160, IgM pos		X
2002-C	19 Jul 2002 HI=10, IgM neg	6 Aug 2002 HI>640, IgM pos		X
2002-D	2 Aug 2002 HI nd, IgM neg	16 Aug 2002 HI>640, IgM pos		X
2002-E	13 Aug 2002 HI<10, IgM neg	13 Sep 2002 HI=80, IgM +/-		X
2002-F	16 Aug 2002 HI<10, IgM neg	6 Sep 2002 HI=320, IgM pos		X
2002-G	13 Sep 2002 HI<10, IgM neg	26 Sep 2002 HI=640, IgM pos		X
2002-H	1 Oct 2002 HI<10, IgM neg	6 Nov 2002 HI=320, IgM pos		X
2007-A	29 Jun 2007 HI<20, IgM +/-	10 Jul 2007 HI=80, IgM pos	X	
2007-B	30 Jul 2007 HI<20, IgM neg	16 Aug 2007 HI=80, IgM pos	X	
2007-C	8 Sep 2007 HI<20, IgM neg	20 Sep 2007 HI=320, IgM pos	X	
2008	29 Jul 2008 HI<10, IgM neg	26 Aug 2008 HI=320, IgM pos	X	

Serum samples drawn from patients 7-36 days before an IgM-positive diagnosis were applied to virus isolation experiments in suckling mice or Vero E6 cell cultures. Patient 2001-B had IgM antibodies already in the first sample but still showed a rise in total antibody level.

Small mammals (IV)

In June 2009 we snap-trapped 17 bank voles *(Myodes glareolus)* and one common shrew *(Sorex araneus)* from two likely sites of human infections in Simo, Finnish Lapland 2008. The mammals were stored in dry ice and -70°C until dissection. We extracted blood from the heart and performed IFA to screen the TBEV-IgG antibody status.

We extracted RNA from the brains and lungs of the mammals by TriPure® Isolation Reagent and performed real-time RT-PCR [Schwaiger and Cassinotti, 2003], modified by us [Tonteri *et al.,* 2011], and 5´-NCR conventional nested RT-PCR [Schrader and Süss, 1999]. Lung and brain suspension from animals which were positive for TBEV-RNA by both RT-PCR methods for at least one organ, had the lowest cycle threshold values in real-time RT-PCR, and had detectable levels of antibodies against TBEV, were applied to virus isolation experiments in suckling mice.

Virus isolation in suckling mice (II, III, IV)

For virus isolation experiments *in vivo*, we used one litter of suckling NMRI mice, aged 0-3 days, for each virus isolation sample. Experimental animal permits were given by State Provincial Office of Southern Finland, decision numbers STU 1385 A, STU 466A, and ESLH-2008-06558/Ym-23.

Reference sequences used for phylogenetic analysis (II, III, IV)

Table 6. Strains used for phylogenetic comparisons in publications II, III and IV.

Virus strain	Acc no	Country	Source	Year	Reference
Kem I	AF091011	Hungary	*I. ricinus*	1952	[Ecker *et al.*, 1999]
Absettarov	AF091005	Russia/St. Petersburg	*H. sapiens*	1951	[Ecker *et al.*, 1999]
Pan	AF091015	Russia/Moscow	*H. sapiens*	1957	[Ecker *et al.*, 1999]
Ljub. I	AF091012	Slovenia	*H. sapiens*	1993	[Ecker *et al.*, 1999]
K23	AF091010	Germany	*I. ricinus*	1975	[Ecker *et al.*, 1999]
Iso 40	AF091009	Switzerland[1]	*I. ricinus*	1975	[Ecker *et al.*, 1999]
ZZ 9	AF091020	Austria	*I. ricinus*	1985	[Ecker *et al.*, 1999]
TBEV 263	U27491	Czech	*I. ricinus*	1987	[Wallner *et al.*, 1995]
Hypr	U39292	Czech	*H. sapiens*	1953	[Wallner *et al.*, 1996]
Neudörfl	U27495	Austria	*I. ricinus*	1971	[Mandl *et al.*, 1988]
235	EF113081	Czech		2006	GenBank
166	EF113079	Czech	*I. hexagonus*		GenBank
433	EF116596	Czech			GenBank
Salem	FJ572210	Germany	*M. sylvaticus*	2006	[Süss *et al.*, 2007]
Kumlinge A 52	X60286	Finland	*I. ricinus*	1959	[Whitby *et al.*, 1993]
Joutseno		Finland	*I. ricinus*	1960	[Uzcátegui *et al.*, unpub.]
Kumlinge-25		Finland	*I. ricinus*	2003	[Uzcátegui *et al.*, unpub.]
Torö-2003	DQ401139	Sweden	*I. ricinus*	2003	[Melik *et al.*, 2007]
Latvia-8110	AJ319583	Latvia	*H. sapiens*		[Lundkvist *et al.*, 2001]
Latvia-9793	AJ319585	Latvia	*H. sapiens*		[Lundkvist *et al.*, 2001]
Lithuania-262	AJ414703	Lithuania	*H. sapiens*	2000	[Mickiené *et al.*, 2001]
KrM215	EU276111	South Korea			[Yun *et al.*, 2009]
KrM216	EU276112	South Korea	*A. agrarius*		[Yun *et al.*, 2009]
Est2546	DQ393779	Estonia	*A. agrarius*	1996	[Golovljova *et al.*, 2004]
Est54	DQ393773	Estonia	*I. persulcatus*	2000	[Golovljova *et al.*, 2004]
Est3476	DQ393776	Estonia	*H. sapiens*	2000	[Golovljova *et al.*, 2004]
Est3535	DQ393774	Estonia	*I. persulcatus*	2001	[Golovljova *et al.*, 2004]
Latvia 1-96	AJ415565	Latvia	*H. sapiens*	1996	[Lundkvist *et al.*, 2001]
Vologda-14-06	FJ214140	Russia	*I. persulcatus*	2006	GenBank
Vologda-15-06	FJ214141	Russia	*I. persulcatus*	2006	GenBank
Vologda-3-75	FJ214143	Russia	*I. persulcatus*	1975	GenBank
Vologda-911-74	FJ214138	Russia	*H. sapiens*	1974	GenBank
Vologda-227-07	FJ214153	Russia	*I. persulcatus*	2007	GenBank
Vologda-658-75	FJ214137	Russia	*H. sapiens*	1975	GenBank
Kurgan-316-07	FJ214151	Russia	*I. persulcatus*	2007	GenBank
Kurgan-264-07	FJ214130	Russia	*I. persulcatus*	2007	GenBank
Yaroslavl-140-98	FJ214146	Russia	*I. persulcatus*	1998	GenBank
Yaroslavl-115-01	FJ214145	Russia	*I. persulcatus*	2001	GenBank
TBEV228	DQ385498	Russia/Novosibirsk	*I. persulcatus*	1981	GenBank
TBEV1467	AY753582	Russia/Novosibirsk			GenBank
Zausaev	AF527415	Russia	*H. sapiens*	1985	[Gritsun *et al.*, 2003a]
Aina	AF091006	Russia	*H. sapiens*	1963	[Ecker *et al.*, 1999]
Vasilchenko	L40361	Russia		1969	[Gritsun *et al.*, 1997]
Z 12	EF566814	Russia	*I. persulcatus*		GenBank

Virus strain	Acc no	Country	Source	Year	Reference
Z 22	EF566816	Russia	*I. persulcatus*		GenBank
Kolarovo-2008	FJ968751	Russia	*I. pavlovskyi*	2008	GenBank
EK-328	DQ486861	Estonia	*I. persulcatus*	1972	[Romanova *et al.,* 2007]
178-79	EF469661	Russia/Irkutsk	*I. persulcatus*	1979	GenBank
886-84	EF469662	Russia/Irkutsk	*M. rufocanus*	1984	GenBank
IR99-1m1	AB049348	Russia/Irkutsk	*I. persulcatus*	1999	[Hayasaka *et al.,* 2001]
IR99-2f13	AB049353	Russia/Irkutsk	*I. persulcatus*	1999	[Hayasaka *et al.,* 2001]
711-84	EU878281	Russia/Buryatia	*M. rufocanus*	1984	GenBank
740-84	EU878282	Russia/Buryatia	*C rufocanus*	1984	GenBank
617-90	EU878283	Russia/Buryatia	*I. persulcatus*	1990	GenBank
205	DQ989336	Russia		1973	[Safronov *et al.,* 1991]
T-blood	AF091019	Russia/Perm	*H. sapiens*	1939	[Ecker *et al.,* 1999]
N132	AF091013	Russia/Vladivostok	*I. persulcatus*	1979	[Ecker *et al.,* 1999]
Crimea	AF091008	Ukraine	*I. ricinus*	1987	[Ecker *et al.,* 1999]
RK1424	AF091016	Latvia	*I. persulcatus*	1977	[Ecker *et al.,* 1999]
Ural-Nina	FJ214119	Russia	*H. sapiens*	1943	GenBank
Glubinnoe/2004	DQ862460	Russia	*H. sapiens*	2004	[Ternovoi *et al.,* 2007]
Primorye-89	FJ906622	Russia	*H. sapiens*	1987	GenBank
Primorye-270	EU816452	Russia	*H. sapiens*	1991	GenBank
Primorye-332	AY169390	Russia	*H. sapiens*	1991	[Leonova *et al.,* 2004]
Dalnegorsk	FJ402886	Russia	*H. sapiens*	1973	GenBank
Kavalerovo	FJ402885	Russia	*H. sapiens*	1985	GenBank
Senzhang	AY182009	China		1953	GenBank
MDJ-01	AY217093	China			GenBank
DXAL-12	EU089977	China			GenBank
DXAL-16	EU089978	China			GenBank
DXAL-18	EU089979	China			GenBank
Kita987/99	AB237192	Japan	*M. rufocanus*		GenBank
Sofjin-HO	AB062064	Russia[2]	*H. sapiens*	1937	[Silber and Soloviev, 1946]
Oshima 5-10	AB062063	Japan	*M. familiaris*	1995	[Takashima *et al.,* 1997]
LIV 369/T2	NC_001809	UK		1963	[Gritsun *et al.,* 1997]
GGEV Vergina	DQ235153	Greece	*I. ricinus*	1969	[Grard *et al.,* 2007]
SSEV 2617	DQ235152	Spain		1987	[Grard *et al.,* 2007]
TSEV TTE80	DQ235151	Turkey		1969	[Grard *et al.,* 2007]
OHFV Kubrin	AY438626	Russia/Omsk	*H. sapiens*	1947	[Li *et al.,* 2004]
OHFV Bogoluvovska	AY193805	Russia		1947	[Lin *et al.,* 2003]
LGTV TP21	AF253419	Malaysia			[Campbell and Pletnev, 2000]
POWV LB	L06436	Canada	*H. sapiens*	1958	[Mandl *et al.,* 1993]
KSIV LEIV 2247	AY863002	Uzbekistan			GenBank

[1]Iso40 is from Switzerland, not from Finland, as we erroneously indicate in Publication II. [2]Sofjin is from Russia, not from Japan, as we erroneously indicate in Publication III. Acc no, GenBank accession number.

Methods

Cloning and expression of TBEV prME (I)

The coding region of prM and E genes of Kumlinge A 52 TBEV-Eur strain was cloned into baculovirus vector pAcYML2 (kindly provided by Dr. Johan Peränen, Institute of Biotechnology, University of Helsinki) and expressed in Sf9 and High Five™ insect cells. The details are described in Publication I.

Reference serological tests (I)

At HUSLAB, the Diagnostic Laboratory of the Hospital District of Helsinki and Uusimaa (formerly Helsinki University Central Hospital HUCH Laboratory Diagnostics) the TBE-IgM test used was Immunozym FSME IgM test (Progen Biotechnik GmbH, Heidelberg, Germany). Total antibodies to TBEV were determined by an in-house hemagglutination inhibition test and DENV IgM antibodies by Dengue Fever Virus IgM Capture ELISA test (Focus Technologies, Cypress, CA). The details are described in Publication I.

TBEV IgM μ-capture assay (I)

The details of the TBEV-IgM μ-capture assay are described in Publication I. Briefly, 96-well plates were coated with goat anti-human IgM (Cappel, West Chester, PA). Patient sera diluted 1:200 in EIA buffer (PBS + 0.5% bovine serum albumin + 0.05% Tween 20) were incubated on wells for 1 h at +37°C. Supernatant from insect cells infected with recombinant baculovirus expressing TBEV-prME genes was diluted 1:5 in EIA buffer but otherwise unhandled and incubated for 1 h at +37°C. Peroxidase-conjugated anti-TBEV-E-MAb 1786 [Niedrig *et al.,* 1994] (6 μg/ml) was incubated for 1 h at +37°C. After each incubation, unbound excess antibodies, antigen and conjugate were washed away. Antibody binding was detected by using tetramethylbenzidine substrate (Sigma, St. Louis, MO). After 15 min incubation at room temperature the reaction was stopped by sulphuric acid and the absorbance measured at 450 nm.

TBEV IgG IFA (I)

The details of the TBEV-IgG IFA are described in Publication I. Briefly, High Five™ cells infected with recombinant baculovirus expressing TBEV-prME genes together with uninfected High Five™ cells were fixed with acetone onto microscope slides. Patient sera diluted in PBS were incubated for 30 min at +37°C. FITC-conjugated anti-human IgG (Jackson ImmunoResearch, West

Grove, PA), diluted 1:50 in PBS, was incubated for 30 min at +37°C. Unbound excess antibodies and conjugate were washed away and the slides were viewed with a fluorescence microscope.

Purification of antigen by ultracentrifugation and sucrose gradient (I)

To define the buoyant density of the recombinant prME antigen we centrifuged the supernatant from Sf9 cells infected with recombinant baculovirus through a sucrose gradient. The supernatant was cleared by centrifugation at 15 900 × *g* for 30 min at +4°C, after which the supernatant was re-centrifuged at 240 000 × *g* for 2 h at +4°C. The pellet was resuspended in 50 µl TEN (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl). The resuspended pellet was centrifuged at 230 000 × *g* for 23 h at +4°C through a 10-60% sucrose gradient. Fractions of 250 µl were diluted 1:16 and the antigenicity of each fraction was studied by EIA. Details are described in Publication I.

Electron microscopy

To concentrate the possibly formed TBEV virus-like particles from High Five™ cells that were infected with recombinant baculovirus expressing TBEV prM and E genes, we first centrifuged the High Five™ supernatant at 11 500 rpm in Sorvall SS-34 rotor for 30 min at +4°C. The supernatant from this run was ultracentrifuged at 25 000 rpm in a Beckman SW 41 rotor (equals to 107 000 × *g)* for 2 h at +4°C in order to remove the baculoviruses, after which the supernatant was re-ultracentrifuged at 33 000 rpm with the same Beckman rotor (equals to 187 000 × *g)* for 2 h at +4°C. This supernatant was ultracentrifuged once more at 41 000 rpm (equals to 288 000 × *g)* for 2 h at +4°C. We resuspended the pellets from each run in 50 µl TEN and stored them at +4°C until use.

The samples from resuspended pellets were fixed onto copper grids (Agar Scientific, Stanstedt, UK) and stained by negative staining method with 2% KPTA (tungstophosphoric acid, pH adjusted to 6.0 by potassium hydroxide). The grids were examined with a Jeol JEM-100 CXII electron microscope.

Hemagglutination test

To test the hemagglutinating capacity of the recombinant TBEV-prME antigen produced in insect cells, we made a dilution series of the insect cell supernatant in borate solution (pH 9.0) including 0.6% bovine serum albumin. Antigen dilutions were incubated in 1:1 mixture with dextrose-gelatin-veronal-washed goose erythrocytes diluted to 0.2% solution in phosphate buffer (pH 6.2 and 6.4) for 30 min at room temperature.

RT-PCR methods (II, III, IV)

Ticks, tick pools, and small mammal samples were screened by TBEV RT-PCR to detect the TBEV RNA in them. We used three methods for screening ticks: (i) a conventional nested NS5-RT-PCR which recognizes TBEV-Eur subtypes [Puchhammer-Stöckl *et al.,* 1995] was modified by us to recognize TBEV-Sib strains as well (Publication II), (ii) a conventional nested 5´-NCR-RT-PCR which recognized TBEV-Eur, TBEV-Sib and TBEV-FE strains [Schrader and Süss, 1999], and (iii) a real-time RT-PCR, which recognizes at least TBEV-Eur and TBEV-Sib subtypes, designed by Schwaiger and Cassinotti, and modified by us: instead of 50 nmol/l of forward primer, 300 nmol/l reverse primer and 200 nmol/l probe [Schwaiger and Cassinotti, 2003] we used 150 nmol/l forward primer, 500 nmol/l reverse primer and 400 nmol/l probe [Tonteri *et al.,* 2011] (Table 7). To compare the sensitivities of these methods, we ran a dilution series of positive control RNA of TBEV-Eur and TBEV-Sib subtypes.

In order to gain more nucleotide sequences we did partial E and NS3 RT-PCRs for the isolated virus strains. For the TBEV-Eur samples, we performed a 1520-nt amplifying E gene nested RT-PCR [Melik *et al.,* 2007], and for the TBEV-Sib and TBEV-FE samples, a more universal 1389-nt amplifying partial E gene RT-PCR (Publication II). For the NS3 segment, we did a reverse transcription reaction using random hexamers (Invitrogen / Life Technologies, Carlsbad, CA) and Expand RT enzyme (Roche) and a pan-flavi NS3 PCR [Billoir *et al.,* 2000; Grard *et al.,* 2007], but with a modified reverse primer 5´-RTTIGCICCCATYTCISHDATRTCIGT.

Table 7. TBEV-RNA screening methods for ticks and small mammals.

Sample panel	RT-PCR screening method			
	NS5-RT-PCR	NS5-RT-PCR mod.	5'-NCR-RT-PCR	Real-time RT-PCR
Kumlinge 2003 (III)	X			
Kokkoal archipelago 2003	X			
Kokkola archipelago 2004 (II)	X	X		
Buryatia 2005 (III)			X	
Lappeenranta 2005 (III)		X	X	
Russian Karelia 2006 (III)		X	X	
Turku archipelago 2007 (III)		X		
Närpiö 2008 (III)		X		
Varkaus 2009			X	X
Simo 2009 ticks (IV)			X	X
Simo 2009 mammals (IV)			X	X
Lappeenranta 2010		X	X	
Kuopio 2010		X	X	

NS5-RT-PCR [Puchhammer-Stöckl *et al.,* 1995]; NS5-RT-PCR mod., modified by us (Publication II); 5´-NCR-RT-PCR [Schrader and Süss, 1999]; real-time RT-PCR [Schwaiger and Cassinotti, 2003], modified by us [Tonteri *et al.,* 2011]. The Roman numerals in parentheses after each panel refer to the publications in this thesis.

Tick species definition (II, III, IV)

Tick species was defined for a panel of 30 ticks from Kokkola archipelago in 2004 [Alekseev *et al.,* 2007], and 198 ticks from Russian Karelia in 2006 by morphological means by Professor Andrey N. Alekseev, Russian Academy of Sciences, St. Petersburg, Russia, and by myself for ticks from Lappeenranta 2005 and 2010, Varkaus 2007, Närpiö 2008, Simo 2009, and Kuopio 2010 according to [Filippova, 1977]. The morphological species definition was further confirmed by *Ixodes* mitochondrial 16S RNA PCR and sequencing [Caporale *et al.,* 1995] for TBEV RNA positive and a few other ticks / tick pools from Kumlinge 2003, Kokkola 2004, Buryatia 2005, and Simo 2009.

IFA for screening TBEV antibodies from wild rodents (IV)

Vero E6 cells infected with Kumlinge A 52 TBEV strain were fixed on a microscope slide with acetone at +4°C for 7 min. The slides were incubated at +37°C for 30 min with blood extracted from the hearts of the rodents, diluted approximately 1:10 in PBS, then with a FITC-conjugated anti-mouse Ig antibody (DakoCytomation A/S, Glostrup, Denmark) at +37°C for 30 min, washed in between each step, and dried. The sample wells were covered with Shannon Immu-Mount mounting medium (Thermo, Pittsburgh, PA) and objective slides, and viewed with Zeiss Axioplan 2 fluorescence microscope, 20 × objective and 10 × oculars.

Virus isolation in mice (II, III, IV)

Nine human serum samples (III) (Table 5), RT-PCR positive ticks / tick pools (II, III, IV) and four RT-PCR positive bank vole heart-lung suspension samples (IV) were further subjected to virus isolation experiments in 0-3 day old suckling NMRI mice. 20 µl of tick / tick pool / rodent lung-brain homogenate diluted 1:1 in Dulbecco's PBS + 0.2% bovine serum albumin, or human sera as such was injected intracranially to each mouse in one litter.

We followed the mice for 14 days or until symptoms appeared, anaesthetized with isoflurane and euthanized. The brains were stored at -70°C until homogenized in 900 µl TriPure® by MagNA Lyser, after which the RNA was extracted according to the manufacturer's instructions.

Virus isolation in cell culture

13 human serum samples (Table 5) were applied to virus isolation experiments in Vero E6 cell cultures. We infected confluent Vero E6 cells in 25 cm^2 cell culture bottle with 150 µl of human serum. After 1 h incubation at +37°C we added 4 ml of minimum essential medium (MEM) supplemented with 2% fetal calf serum, streptomycin, penicillin, and glutamine, and incubated the cells at +37°C at 5% CO_2 for two weeks. Then the cells were collected and together with fresh Vero E6 cells changed to a 75 cm^2 cell culture bottle and incubated with MEM supplemented with 10% fetal calf serum, streptomycin, penicillin, and glutamine at +37°C at 5% CO_2 until the cells were confluent. The media was changed to MEM with 2% fetal calf serum, streptomycin, penicillin, and glutamine, and the incubation at +37°C at 5% CO_2 was continued until day 14 after infection, after which two thirds of the cells were washed and fixed (7 min acetone at +4°C), together with non-infected Vero E6 cells, to objective slides for IFA staining, and passaging was repeated for the rest of the cells.

Sequencing (I, II, III, IV)

Positive amplicons from ticks and rodents as well as from virus isolation experiments were sequenced using the same primers as for PCRs. The partial E genes were sequenced from the middle as well by left primer 5´-CGCAAAACTGGAATAA CGC and a right primer 5´-CATCTTGACAGCGTGAGGAG. Sequencings were done by cycle sequencing with Big Dye Terminator kit (version 3.1) by Applied Biosystems (Foster City, CA). Reactions were run on ABI 3130*xl* capillary sequencer according to the manufacturer's instructions.

Sequence analysis and phylogeny (II, III, IV)

The sequences were manually aligned with published sequences from global isolates of TBEV deposited in GenBank to perform phylogenetic analyses (Table 6). For publication II, partial E sequences were aligned using ClustalX [Thompson *et al.,* 1997] and phylogenetic trees estimated using the maximum likelihood method. For making trees, we used Modeltest [Rodriguez *et al.,* 1990]. All these analyses were undertaken using PAUP* [Swofford, 2000]. For publications III and IV, we used partial E and NS3 sequences, because the latter are estimated to give trees that most resemble the full-length trees [Billoir *et al.,* 2000; Cook and Holmes, 2006]. Sequences were handled using BioEdit [Hall, 1997] and phylogenetic trees of both E and NS3 sequences were estimated using the Bayesian approach implemented in the program BEAST [Drummond and Rambaut, 2007].

Results and discussion

μ-capture IgM EIA for acute TBE diagnostics (I)

Expression of recombinant TBEV antigen

We expressed the prM and E genes of TBEV-Eur strain Kumlinge A 52 ([Brummer-Korvenkontio *et al.,* 1973]; GenBank accession number AY268437) with the baculovirus expression system in insect cells. The cloned sequence began 15 amino acids before the predicted signal sequence of prM, contained the complete prM and E sequences including the hydrophobic transmembrane regions, and 30 amino acids from the amino terminus of NS1 (Figure 1 in Publication I). Cellular proteases should cleave the expressed recombinant polypeptide to prM and E proteins (see Figure 3B) to produce virus-like particles (VLPs). The recombinant antigen was found both in the infected insect cells and secreted to the insect cell culture medium, as seen by immunofluorescence staining of the infected High Five™ cells by acute and convalescent phase TBE patient sera (Figure 5 in Publication I) and by immunoblotting the infected Sf9 cell lysates and the cell culture supernatant by monoclonal antibodies [Niedrig *et al.,* 1994] against TBEV E protein (Figure 2 in Publication I).

We assume that the antigen was secreted from the cells in the form of VLPs. This assumption was supported by the following experiment. When the concentrated cell culture supernatant was centrifuged through a sucrose gradient, a peak in antigenicity was seen at 36% w/v sucrose, corresponding to buoyant density of 1.15 g/cm^3 (Figure 3 in Publication I), which is the same value as that of JEV recombinant VLPs [Hunt *et al.,* 2001] and very close to what has been found for TBEV VLPs produced in mammalian cells, 1.14 g/cm^3 [Schalich *et al.,* 1996]. TBEV E protein is a glycoprotein with a single N-linked glycosylation site, and the different glycosylation pattern in mammalian and insect cells [Jarvis, 2003] could possibly affect the buoyant density. For whole virions which include the nucleocapsids in addition to the glycoproteins, the buoyant density is 1.19 g/cm^3 [Russell *et al.,* 1980; Schalich *et al.,* 1996]. Furthermore, in electron microscopy we saw entities that according to their shape and size, estimated as 30-40 nm, could be VLPs (Figure 11). However, these particles were not numerous even in the ultracentrifugation-concentrated supernatant, and to confirm whether they truly represent VLPs would require more extensive studies.

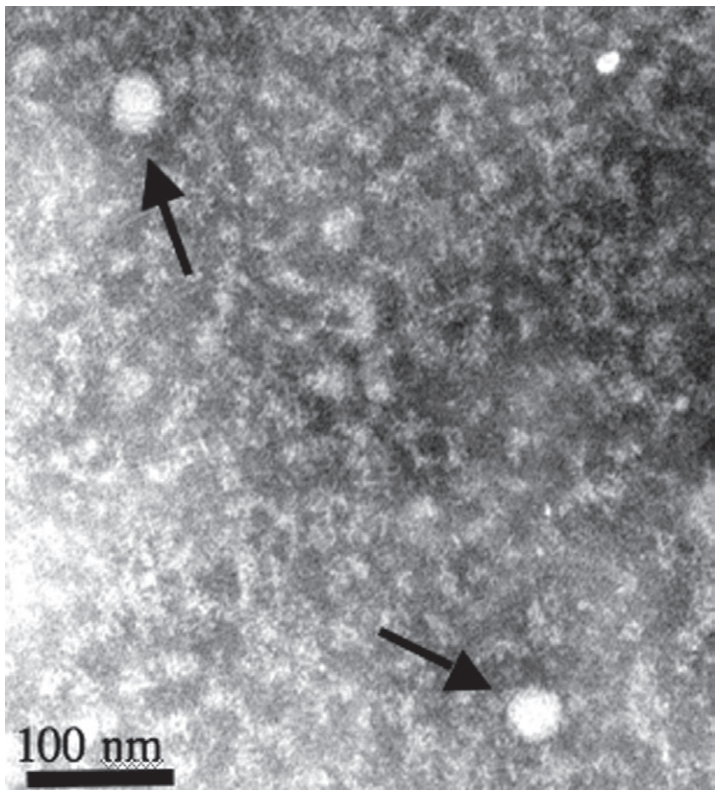

Figure 11. Possible virus-like particles. The supernatant of bac-TBEV-prME infected insect cells was concentrated and viewed by electron microscopy.

The recombinant antigen did not agglutinate goose erythrocytes although flaviviruses have hemagglutination activity, maybe because it was expressed in insect cells. The glycoprotein E of TBEV acts as a viral hemagglutinin [Chambers *et al.,* 1990] and the carbohydrate residues are possibly involved in hemagglutination.

IgG IFA

Although not the actual focus of this study, we tested the potential of the recombinant antigen for use in IgG diagnostics in an immunofluorescence format, described in Publication I. Briefly, all acute-phase sera (IgM positive, n = 48) were also detected by IgG IFA based on recombinant baculovirus-infected High Five™ cells, and 95% of negative (HI titer < 10, n = 88) were negative in IgG IFA. However, as only 7 out of 12 sera considered old-immunity samples (HI titer ≥ 10, IgM negative) were recognized by the recombinant antigen in IgG IFA format, the sensitivity seemed not to be appropriate for diagnostic use or seroprevalence studies.

μ-capture IgM EIA

One of the main aims in this thesis was to improve the diagnostics of acute TBE by developing a test that would be based on a safe, non-infectious antigen. We used the diluted and otherwise unhandled supernatant from the cells expressing TBEV prM and E genes directly as an antigen in an IgM EIA test.

We developed the IgM EIA test essentially in a similar format as described previously for acute Puumala hantavirus infection diagnostics [Vapalahti *et al.,* 1996]. It is a *μ*-capture EIA format, where the EIA wells are coated with anti-human IgM antibody which binds all IgM antibodies present in serum or cerebrospinal fluid samples, and then the specific antibodies are detected by adding an antigen whose binding is detected by a specific peroxidase-conjugated monoclonal antibody. The EIA test development and its evaluation by 100 TBEV-IgM negative, 50 TBEV-IgM positive and seven DENV-IgM positive serum samples are described in Publication I. Two of the positive samples were positive for rheumatoid factor. That is an autoantibody against the IgG Fc fragment, that often interferes with viral diagnostics [Salonen *et al.,* 1980]. An IgM-EIA in a capture format should tolerate rheumatoid factor better than an indirect EIA [Bendig and Molyneaux, 1996]. We believe the two rheumatoid factor positive samples may have been false positives in the reference test and thus we excluded them from the specificity and sensitivity calculations. We have later evaluated the *μ*-capture IgM EIA test with additional 418 TBEV-IgM negative and six positive samples (Table 8). With this panel (525 negative and 54 positive sera), when the two rheumatoid factor-positive samples were excluded, the specificity and sensitivity of our *μ*-capture IgM EIA were 99% and 100%, respectively.

Table 8. Comparison of two IgM EIA tests for TBEV infection.

		Progen IgM EIA		
		Positive	Negative	Total
μ-capture IgM EIA	Positive	54	4	58
	Negative	0	521	521
	Total	54	525	579

Since its development, that is in 2003, the *μ*-capture IgM EIA based on secreted baculovirus-expressed recombinant TBEV prME antigen has been used as the primary test for routine diagnostics of acute TBE in the diagnostic laboratory of HUSLAB (Diagnostic Laboratory of the Hospital District of Helsinki and Uusimaa), Helsinki, which is one of the two laboratories in Finland currently performing TBE diagnostics. Approximately 3500 samples have been tested so far, including twelve control samples from an external quality-assurance program organized by the European Network for the Diagnostics of "Imported" Viral Diseases (www.enivd.org) in 2005, of which our IgM EIA correctly diagnosed all [Niedrig *et al.,* 2007].

Coexpression of TBEV prM together with E leads to generation of secreted VLPs [Allison *et al.,* 1995] where the E protein, which is the major antigen, is folded properly [Schalich *et al.,* 1996]. Recombinant VLPs are superior antigens in TBE serology [Heinz *et al.,* 1995], apparently because they are properly folded and thus very much resemble the real virions, and because they are secreted which circumvents the need of potentially harsh purification methods. The benefit of secretion was seen when recombinant TBEV antigen was expressed as VLPs in mammalian cells: both the specificities and the sensitivities of IgG and IgM EIA tests improved when the recombinant antigen in a cell culture lysate [Yoshii *et al.,* 2003] was replaced by the same but secreted antigen [Obara *et al.,* 2006].

VLPs produced in mammalian cell lines have been described to be adequate for diagnosis of JEV [Konishi *et al.,* 1996, tested for vaccinees only; Hunt *et al.,* 2001], WNV [Davis *et al.,* 2001; Hogrefe *et al.,* 2004], and for TBEV [Yoshii *et al.,* 2003; Obara *et al.,* 2006] infections. To our knowledge we were the first to publish a diagnostic test for flavivirus infection based on recombinant antigen produced in insect cells. Since then reports of recombinant vaccine candidates for WNV [Qiao *et al.,* 2004; Bonafe *et al.,* 2009] and DENV-2 and JEV [Kuwahara and Konishi, 2010] produced in insect cell systems have been published.

Mammalian and insect cell expression methods have both their advantages and disadvantages. Protein glycosylation differs in mammalian and insect cells [Jarvis, 2003] and in some cases it may affect the folding or antigenicity of the recombinant protein. On the other hand, insect cells are in general easier to cultivate than mammalian cells [Kuwahara and Konishi, 2010]. Insect cell cultures produced 10 to 100-fold higher amounts of DENV-2 and JEV VLPs, respectively, than mammalian cells [Kuwahara and Konishi, 2010] and they can be modified from monolayers to suspension cultures which is a benefit in large scale antigen production systems.

TBEV is a biosafety level 3 (or 4, depending on national regulations) classified pathogen, and thus its cultivation requires highly specialized laboratories. Conventional serological tests (e.g. IFA, HI, EIA, NT) require the use of cell-cultured TBEV antigen [Yoshii *et al.,* 2003; Sonnenberg *et al.,* 2004], and apparently all commercially available EIA kits are based on inactivated TBE virus [Marx *et al.,* 2001; Niedrig *et al.,* 2001, 2007; Yoshii *et al.,* 2003; European Network for the Diagnostics of "Imported" Viral Diseases, 2010]. Recombinant non-infectious antigens circumvent the need for mass purified biosafety level 3 virus. They do not need to be inactivated which would possibly affect the antigenic properties e.g. by disturbing the conformation of epitopes [Obara *et al.,* 2006]. Thus non-infectious antigens are safer, easier to handle and cheaper, and in some cases closer to wild-type antigen than inactivated ones.

Molecular epidemiology of TBEV (II, III, IV)

TBE is endemic in a zone from central and eastern Europe through Siberia to the Far East (Figure 6). In Finland, TBE is endemic in the Åland Islands, Turku archipelago, Kokkola archipelago, Lappeenranta-Parikkala region in southeastern Finland [Tuomi and Brummer-Korvenkontio, 1965], and Isosaari island in Helsinki archipelago [Han *et al.,* 2001] (Figure 9A). Recently sporadic human cases appeared in Närpiö (2007 and 2010), Varkaus (2008), Simo (2008 and 2009), around Kuopio (2009 and 2010), and Kotka (2010) (Figure 9B). We collected ticks by flagging from all these areas (samples from Kotka have not yet been analyzed and are not included in this thesis). We also collected ticks from two locations in Russia, both endemic for TBE: the Republic of Karelia in north-western Russia, in an area where *I. ricinus* and *I. persulcatus* ticks overlap, and the Republic of Buryatia in eastern Siberia, in the middle of the range of *I. persulcatus* distribution (Figure 6; Figure 1a in Publication III).

The detailed results of these tick collections are described in Publication II (Kokkola archipelago), IV (Simo) and III (other tick panels). The main findings include: 1) *I. persulcatus* was found to be distributed along the western coast of Finland, but still only *I. ricinus* in the southern, central and eastern TBE-endemic foci; 2) TBEV-Eur isolation from southern Finnish TBE endemic foci; 3) TBEV-Sib isolation from Kokkola archipelago and Russian Karelia; 4) TBEV-FE isolation from Buryatia; 5) detection of TBEV in Simo in Finnish Lapland, a recently established and the northernmost TBE endemic focus in the world; 6) *I. persulcatus* unorthodoxly carried TBEV-Eur in Simo; and 7) TBEV prevalence was approximately 1% in questing ticks in the majority of these TBE-endemic sites independently of the RT-PCR method, geographical area or year of sampling. The results are summarized in Table 9.

In addition to ticks, we isolated four TBEV-Eur strains from bank voles *(Myodes glareolus)* from Simo and two TBEV-Eur strains from Finnish acute TBE patients. Both patients had spent time in the known TBE-endemic areas in the archipelagos of south-western Finland.

Tick panel	Location	Tick species	Ticks/ pools	Positive in NS5-RT-PCR[1]	Positive in 5´-NCR-RT-PCR[2]	Positive in real-time RT-PCR[3]	Positive in virus isolation	Virus subtype	Prev.	Ref.
Kumlinge 2003	60°16´N, 20°46´E	*I. ric*	454 / 45	4	ND	ND	3	Eur	0.9	III
Kokkola archipelago 2003	63°55´N, 22°48´-23°04´ E	ND	139 / 15	0	ND	ND	NA	NA		
Kokkola archipelago 2004	63°55´N, 22°48´-23°04´ E	*I. per*	1181 / 122	13	ND	ND	11	Sib	1.1	II
Buryatia 2005	52°55´N, 106°15´-107°41´E	*I. per*	296	ND	2 (3)[5]	ND	1	FE	0.7 (1.0)[5]	III
Isosaari 2005	60°06´N, 25°03´E	*I. ric*	96 / 11	1	1	ND	1	Eur	1.0	III
Lappeenranta 2005	61°03´N, 28°11´E	*I. ric*	292 / 29	0 (2)[5]	0	ND	0	NA	0 (0.7)[5]	III
Russian Karelia 2006	61°47´N, 34°20´E	Both[4]	198	1	2	ND	2	Sib	1.0	III
Turku archipelago 2007	60-61° N, around 22° E	*I. ric*	1039 / 315	1	ND	ND	1	Eur	0.1	III
Närpiö 2008	62°28´N, 21°20´E	*I. per*	36	0	ND	ND	NA	NA	0	III
Varkaus 2009	62°19´N, 27°50´E	*I. ric*	10 / 4	ND	0	0	NA	NA	0	
Simo 2009	65°40´N, 24°54´E	*I. per*	97 / 51	ND	2	6	2	Eur	2.1 (6.2)[5]	IV
Lappeenranta 2010	61°03´N, 28°11´E	*I. ric*	101 / 11	ND	0	0	NA	NA	0	
Kuopio 2010 (Jännevirta)	62°58´N, 27°49´E	*I. ric*	33 / 4	ND	0	0	NA	NA	0	

Table 9. Tick panels studied for TBEV. [1]NS5-RT-PCR was done according to Puchhammer-Stöckl and others [1995] for Kumlinge 2003 and Kokkola archipelago 2003 and 2004 tick panels, and by a modified version described in Publication II for Kokkola archipelago 2004, Isosaari 2005, Lappeenranta 2005, Russian Karelia 2006, Turku archipelago 2007, and Närpiö 2008 panels. [2]5´-NCR-RT-PCR was done according to Schrader and Süss [1999] for Buryatia 2005, Isosaari 2005, Lappeenranta 2005 and 2010, Russian Karelia 2006, Varkaus 2009, Simo 2009, and Kuopio 2010 panels. [3]Real-time RT-PCR was done according to Tonteri and others [2011], modified from a method by Schwaiger and Cassinotti [2003], for tick panels from Varkaus 2009, Simo 2009, Lappeenranta 2010, and Kuopio 2010. [4]Among the 198 ticks from Russian Karelia 2006, five were *I. ricinus* and the rest *I. persulcatus*. [5]Number of positives in parentheses when we were not able to confirm all of them by sequencing or virus isolation. Prev., prevalence. Ref., number of publication in this thesis. *I. ric, Ixodes ricinus*. *I. per, Ixodes persulcatus*. ND, not determined. NA, not applicable.

Performance of RT-PCR methods in screening TBEV in ticks

We studied tick samples collected from a wide geographical range. Most of our panels were studied by at least two RT-PCR methods (Tables 7 and 9). We used two conventional RT-nested PCR methods and one real-time RT-PCR method. The detection level of both conventional nested RT-PCR methods is about 100-1000 copies of TBEV RNA in cerebrospinal fluid and serum samples [Puchhammer-Stöckl *et al.,* 1995; Schrader and Süss, 1999], and 10 focus forming doses of TBEV in tick samples [Han *et al.,* 2001]. A real-time RT-PCR method has been reported to detect less than 10 copies of TBEV RNA [Schwaiger and Cassinotti, 2003] and being highly specific and sensitive for patient and tick suspension samples [Donoso Mantke *et al.,* 2007a, 2007b].

We ran comparative dilution series PCRs for TBEV-Eur (Kumlinge A 52) and TBEV-Sib (Kokkola-118) control RNAs. It seemed that the real-time RT-PCR was at least 10-fold more sensitive than the modified NS5-RT-PCR or the 5´-NCR-RT-PCR for both TBEV-Eur and TBEV-Sib. This was supported by our finding of TBEV RNA positive ticks and rodents in Simo, 2009: 2% of ticks were positive by 5´-NCR-RT-PCR and 6% by the real-time RT-PCR. As many as 15/17 bank voles were positive for either lung or brain by the real-time RT-PCR and four of them by the conventional 5´-NCR-RT-PCR method. We have not confirmed all the real-time RT-PCR positives by e.g. virus isolation trials. However, the rodents were sampled during tick activity time in summer. TBEV RNA can persist in rodents for months [Achazi *et al.,* 2011; Tonteri *et al.,* 2011] thus by late June the rodents may have accumulated it from several tick bites. For the nested RT-PCR methods we did not observe significant differences in sensitivities (Table 9).

Kokkola archipelago 2003 tick panel was studied by the NS5-RT-PCR [Puchhammer-Stöckl *et al.,* 1995], which is rather specific for TBEV-Eur. None of the 139 ticks were positive. In the following year we collected 1181 ticks from the same archipelago and repeated the same RT-PCR for these, again all negative, after which we designed a new primer for the RT-PCR to detect TBEV subtypes more widely, and ran the ticks from 2004 again. With the modified primer 13 tick pools from Kokkola archipelago 2004 were TBEV RNA positive, and they turned out to be TBEV-Sib subtype (Publication II). It is possible that there could have been TBEV-Sib positives in the 2003 panel from the same area as well which, however, were not recognized by the original RT-PCR method.

TBEV prevalence in ticks

In many of our tick panels from TBEV endemic areas the prevalence of TBEV RNA in field-collected questing ticks was about 1%, independently of the year or season of sampling or the geographical area (Table 9; Publications II and III). We did not find any positives from Närpiö 2008 (36 ticks, Publication III), Varkaus 2009 (10 ticks, unpublished), Lappeenranta 2010 (101 ticks, unpublished)

or Kuopio 2010 (33 ticks, unpublished), possibly because the panels were so small. Lappeenranta-Parikkala area is a region with repeatedly reported human TBE cases and an area where cows had TBE antibodies already in the 1960s [Tuomi and Brummer-Korvenkontio, 1965]. TBEV-Eur strain Joutseno was isolated from a pool of *I. ricinus* from the region in 1960 [Brummer-Korvenkontio *et al.,* 1973].

According to a German definition of an endemic area, at least five cases within five years or two cases within a year [Süss *et al.,* 2004], Närpiö can probably be considered a true TBE-endemic focus. Four human cases (one in 2007 and three in 2010; Teija Korhonen, National Institute for Heath and Welfare, personal communication) have been reported from the region. From Varkaus only one and from Kuopio region two human cases have ever been reported. These sites (Varkaus and Kuopio) may or may not be TBE endemic. Possibly TBEV circulates or has circulated there unnoticed in nature, but another scenario could be that a host animal (e.g. a bird) has brought to the area only one or very few TBEV-infected ticks, which bit the human victim and infected him/her without TBEV truly being established at that location.

The low prevalence (0.1%) in Turku archipelago (Publication III) where human TBE cases are reported every year, and where cows had TBEV antibodies in the 1960s [Tuomi and Brummer-Korvenkontio, 1965] compared to other TBE-endemic areas may have been influenced by different selection of the tick-collection sites. In other Finnish tick collection sites we used the information obtained by the National Institute for Health and Welfare when they interviewed the TBE patients, or in Kokkola archipelago by Dr. Tapani Tikkakoski (Table 1 in Publication II), in Russian Karelia by Dr. Nataliya Subbotina, and in Buryatia by Dr. Galina B. Murueva, to concentrate our sampling very focused to the likely sites of infections, but in Turku archipelago the tick collection was more random and may have included islands where TBEV is not circulating. The transmission cycle of TBEV-Eur is fragile and dependent on microclimatical and ecological factors [Randolph *et al.,* 2000], thus TBEV risk is not necessarily equally distributed within the islands [Brummer-Korvenkontio *et al.,* 1962]. Also in the 1950s and 1960s the TBEV prevalence in ticks in the archipelagos of Åland and Turku varied between 0.3-3% on different islands [Brummer-Korvenkontio *et al.,* 1973].

The prevalence of TBEV in field-collected questing *I. ricinus* in TBE-endemic areas in Europe is usually 0.2-5% [Han *et al.,* 2001, 2005; Süss *et al.,* 2002, 2006; Golovljova *et al.,* 2004; Carpi *et al.,* 2009; D'Agaro *et al.,* 2009] depending on the location and sampling time. Exceptionally high prevalence of 14.3% with unusually high partial 5´-NCR sequence variation was reported in *I. ricinus* nymphs and adults in Belp in Switzerland in 2004 [Casati *et al.,* 2006], but such findings have not, to my knowledge, been confirmed by other studies. TBEV prevalence in engorged *I. ricinus* collected from humans is higher than in questing ones [Süss *et al.,* 2004, 2006; Klaus *et al.,* 2009; Belova *et al.,* 2011].

In our panel, two out of 193 *I. persulcatus* from Russian Karelia, and three out of 296 from the Republic of Buryatia, carried TBEV (Table 9; Publication III), and gave the same (approximately 1%) prevalence as in most of the endemic

foci in Finland, including Kokkola archipelago, where we have detected only *I. persulcatus*. Much higher TBEV prevalence data have been obtained from *I. persulcatus* ticks from Russia. The prevalence in Russian Karelia 1993-2004 increased from 8 to 15.2% [Bespyatova *et al.,* 2006]. An even higher prevalence of 46% in *I. persulcatus* has been reported in western Siberia near Novosibirsk in 2001 [Morozova *et al.,* 2002], however, without any confirmatory assays. In Pre-Ural region in Russia the prevalence in *I. persulcatus* varied between 10.9 and 38.7% in 1993-1998, and among a small sample of 30 ticks in 1997 was as high as 77% [Korenberg and Kovalevskii, 1999]. TBEV prevalences in our ticks by the methods we used were not even close to these. From the publications we have obtained it is not always possible to trace the detection methods, which may affect the results. In Irkutsk province (next to Buryatia in Siberia) the prevalence in ticks studied by EIA was 2.9-5.4% in free-living ticks and 10% in ticks fed on humans, but rose to 20-40% if studied by infecting mice or cell culture [Süss, 2003]. However, the prevalence may indeed vary enormously in different years even at the same location. For instance in Latvia, TBEV prevalence varied between 0-37.3% in 1995-2002, detected by EIA [Bormane *et al.,* 2004]. Our panels have been studied in the same laboratory by the same methods and thus are comparable with each other. We confirmed most of our RT-PCR positive tick samples by sequencing the amplicons and/or by virus isolation (Tables 9 and 10).

TBEV prevalence in bank voles in Simo, 2009

We trapped 17 bank voles and one shrew from Simo in June 2009. As many as fifteen of the bank voles were positive for TBEV RNA by the real-time RT-PCR [Schwaiger and Cassinotti, 2003], modified by us [Tonteri *et al.,* 2011], and four by 5´-NCR-RT-PCR [Schrader and Süss, 1999]. Even with the less sensitive conventional RT-PCR, the prevalence in rodents was much higher than in ticks. We isolated four TBEV strains from the rodents. One might want to consider sampling rodents instead of ticks when aiming to study presence of TBEV or to obtain TBEV strains e.g. for molecular epidemiology studies.

Cell culture isolation

We were not successful in isolation trials in Vero E6 cultures. However, the human serum samples applied for cell culture isolation experiments had been stored at -20°C for years (Table 5) which is suboptimal for virus isolation experiments, and when the same samples were applied for both cell culture and suckling mouse isolations, they were negative in both. To avoid the use of live mice, the cell culture trials, including blind passages, should be continued, and new cell lines should be tested.

Genetic analysis of TBEV strains

The detailed results of genetic analysis of the isolated TBEV strains are described in Publications II, III, and IV, and the TBEV strains isolated are listed in Table 10.

The TBEV-Eur strains isolated from ticks from Kumlinge in the Åland Islands, Turku archipelago, Isosaari island in the archipelago of Helsinki, from TBE patients who had been spending time in southwestern archipelagos of Finland, and from ticks and bank voles from Simo were all very closely related. Within 1183 nt from the E gene the TBEV-Eur strains described in this study were at least 96% identical to each other and to other TBEV-Eur sequences found in GenBank. No major geographical clustering was observed, which is very typical of TBEV-Eur strains. Based on full open reading frame sequences TBEV-Eur strains seem to have diverged from each other only about 300 years ago, whereas TBEV-Sib and TBEV-FE strains have diverged about 1000 and 2000 years ago, respectively [Uzcátegui *et al.,* unpublished]. However, strains isolated from Kumlinge in 2003 were identical or almost identical to each other and Kumlinge A 52 strain isolated from the same island in 1959 [Brummer-Korvenkontio *et al.,* 1973] and the closest relative was from the archipelago of Turku (Korppoo-259). Similarly, the isolates from Simo were at least 99% identical to each other. As discussed in Publication III, this observed small-scale clustering may indicate a single event in introducing the TBEV strains to a certain location. In contrast to what has been speculated before [Wahlberg *et al.,* 2006], although the climate in the northern limits of TBE endemic area in Europe may seem harsh for ticks or virus strains to survive, we believe that TBEV is established to circulate in Åland and Turku archipelagos independently without the need of new TBEV introductions each summer. The same probably applies to Isosaari in the archipelago of Helsinki although previous data from there are much more limited than from Åland. The partial NS5 sequence of 150 nt from *I. ricinus* pool from Isosaari from 1996 [Han *et al.,* 2001] was identical to our Isosaari-5 from 2005.

No previous data of TBEV in Simo are available, as probably TBEV has only recently been introduced there.

The Siberian subtype strains vary more and can actually be divided into two geographical clusters: one which includes TBEV-Sib strains isolated from Siberia and another one which includes the TBEV-Sib strains isolated from Europe [Golovljova *et al.,* 2008]. TBEV-Sib strains described in this thesis include 11 strains isolated from Kokkola archipelago, which were at least 98% identical to each other on the nucleotide level within 1225 nt, and two strains isolated from Russian Karelia, which were 96% identical to each other within 1223 nt from the E gene. All these carried the signature amino acids for “Baltic-Siberian” TBEV E protein [Golovljova *et al.,* 2008]. The Russian Karelian strains were surprisingly divergent considering the fact that they were isolated from the same forest on the same day, and clustered differently in the phylogenetic tree based on partial E gene (Figure 1b in the Publication III). The co-circulation of such divergent

strains indicates either multiple introductions of TBEV, or that the virus has been circulating in Russian Karelia for time long enough to diverge so much. More sequence data are needed to conclude for how long.

Table 10. GenBank accession numbers of nucleotide sequences amplified by PCR or isolated from positive tick, rodent and human samples.

Strain	Year	Source	Type	Partial 5'NCR	Partial E	Partial NS3	Partial NS5
Kumlinge-24	2003	*I. ric* pool	Eur		HM051166		HM051176
Kumlinge-25[1]	2003	*I. ric* pool	Eur				
Kumlinge-38	2003	*I. ric* pool	Eur	HM051162	HM051167	HM051188	HM051177
Kumlinge-39	2003	*I. ric* pool	Eur		HM051168		HM051178
Kokkola-4	2004	*I. per* pool	Sib				DQ451297
Kokkola-8	2004	*I. per* pool	Sib		DQ451286		DQ451298
Kokkola-9	2004	*I. per* pool	Sib		DQ451287		DQ451299
Kokkola-25	2004	*I. per* pool	Sib		DQ451288		DQ451300
Kokkola-26	2004	*I. per* pool	Sib		DQ451289		DQ451301
Kokkola-39	2004	*I. per* pool	Sib		DQ451290		DQ451302
Kokkola-79	2004	*I. per* pool	Sib		DQ451291		DQ451303
Kokkola-81	2004	*I. per* pool	Sib		DQ451292		DQ451304
Kokkola-84	2004	*I. per* pool	Sib		DQ451293	HM051186	DQ451305
Kokkola-85	2004	*I. per* pool	Sib				DQ451306
Kokkola-86	2004	*I. per* pool	Sib		DQ451294		DQ451307
Kokkola-102	2004	*I. per* pool	Sib		DQ451295	HM051187	DQ451308
Kokkola-118	2004	*I. per* pool	Sib		DQ451296		DQ451309
Isosaari-5	2005	*I. ric* pool	Eur		HM051169	HM051190	HM051179
Buryatia-169	2005	*I. per*	FE	HM051165	HM051175	HM051189	
Buryatia-171	2005	*I. per*	FE	HM051164			
Karelia-94	2006	*I. per*	Sib	HM051161	HM051173	HM051184	
Karelia-108	2006	*I. per*	Sib	HM051160	HM051174	HM051185	HM051180
Korppoo-259	2007	*I. ric* pool	Eur	HM051163	HM051170	HM051181	
FinHuman-2007[2]	2007	*H. sapiens*	Eur		HM051171	HM051182	
FinHuman-2008[3]	2008	*H. sapiens*	Eur		HM051172	HM051183	
Simo-2	2009	*M. gla*	Eur		HQ228016		
Simo-5	2009	*M. gla*	Eur		HQ228017	HQ228022	
Simo-7	2009	*M. gla*	Eur		HQ228018	HQ228023	
Simo-9	2009	*M. gla*	Eur		HQ228019	HQ228024	
Simo-38	2009	*I. per* pool	Eur		HQ228014	HQ228020	
Simo-48	2009	*I. per* pool	Eur		HQ228015	HQ228021	

Isolated strains are in bold. *I. ric, Ixodes ricinus*. *I. per, Ixodes persulcatus*. *M. gla, Myodes glareolus*.
[1]The complete open reading frame of Kumlinge-25 has been sequenced [Uzcátegui *et al.,* unpublished].
[2]FinHuman-2007 is the patient 2007-B and [3]FinHuman-2008 is the only patient from 2008 in Table 5.

I. ricinus and I. persulcatus distribution

We collected ticks from all sites in Finland known or suspected to be endemic for TBE and in two Republics in Russia, Karelia and Buryatia, to study the molecular epidemiology of TBEV. As a by-product of this research we also studied *I. ricinus* and *I. persulcatus* distribution in these areas. As we only concentrated to sites where human TBE cases have been reported, the tick distribution data

are by no means conclusive information of the true tick species distribution in Finland, Karelia or Buryatia in general.

We collected a total of 3972 questing ticks by flagging. The tick species was not determined for 139 ticks collected in Kokkola 2003, but because *I. persulcatus* was found there in 2004 (Figure 12), it is likely they were also *I. persulcatus*. For the other tick panels, the species definition was done for a few samples by morphology, and for the TBEV RNA positive ticks and few other samples the species was confirmed by mtDNA sequencing [Caporale *et al.,* 1995]. For the ticks from Russian Karelia, Närpiö, Kuopio and Varkaus the species was defined for every specimen. Indeed, in Russian Karelia, there were 5 *I. ricinus* and 193 *I. persulcatus* among the 198 ticks. Thus *I. ricinus* and *I. persulcatus* can, at least in some cases, be questing in the same area. However, Gunnar Hasle has speculated that a new tick species would probably not establish a viable colony in an area already occupied by another species [Hasle, 2011]. In areas in eastern Europe where *I. ricinus* and *I. persulcatus* overlap, their distribution is still separated to microclimatic niches [Lindgren and Jaenson, 2006]. The five *I. ricinus* adults we found among the 198 ticks in Russian Karelia in 2006 are probably just incidental introductions. *I. ricinus* was not found among 2430 ticks (1804 *I. persulcatus* and 626 *I. trianguliceps)* in Russian Karelia very close to our sampling sites, 60 km north of Pedrozavodsk, in 1995-2003 [Bespyatova *et al.,* 2006].

For the rest of the sampling sites we only have evidence of one tick species, either *I. ricinus* (southern, eastern and central Finland), or *I. persulcatus* (central and northern western coast of Finland, and Buryatia).

If we assume that all the ticks in a given panel are of the same species (except for Russian Karelia), our blanket dragging yielded 1328 *I. ricinus* nymphs, 4 larvae, and 697 adults, but only 96 *I. persulcatus* nymphs and 1707 adults. Larvae were not collected intensively due to the fact that they have not yet fed and thus are less likely to be positive for TBEV; besides, it is sometimes hard to reach the lower parts of vegetation, where the larvae predominantly quest, with the flagging method [Knap *et al.,* 2009]. *I. persulcatus* nymphs and larvae are active at night [Anderson and Magnarelli, 2008], which probably partly explains the small amount of *I. persulcatus* nymphs (96) compared to adults (1707). However, we rarely went to same places again and did not register weather conditions, and thus these data do not allow conclusions about the possible differences in the activities of the two tick species.

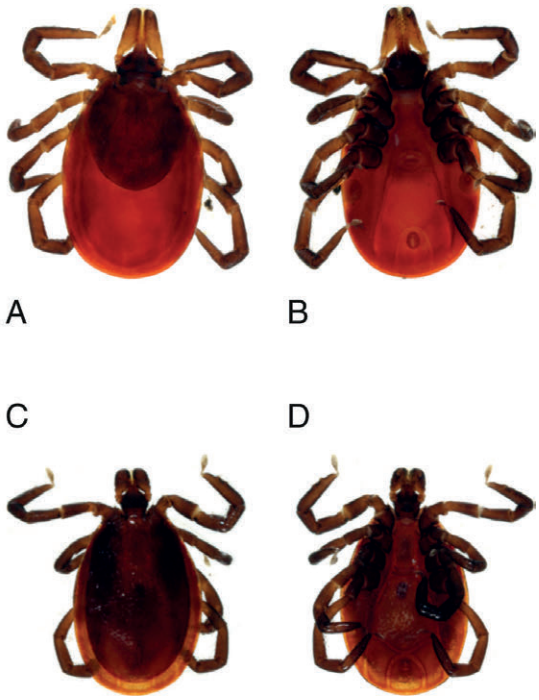

Figure 12. *I. persulcatus* female and male from Kokkola archipelago 2004. A. female, dorsal; B. female, ventral; C. male, dorsal; D. male, ventral view.

I. ricinus and I. persulcatus distribution in Finland

As expected, the ticks collected from southwestern Finland, the Åland Islands, Turku archipelago, and Helsinki archipelago were *I. ricinus* (Publication III; Figure 13). However, to our surprise, in 2004, we found that the tick species in Kokkola archipelago is actually *I. persulcatus* (Figure 12) (Publication II). Later we encountered *I. persulcatus* on other sites along the western coast of Finland as well, in Närpiö in 2008 (Publication III) and in Simo in 2009 (Publication IV) (Figure 13). Still, according to our observations, ticks collected from more eastern Finland – thus closer to the known *I. persulcatus* distribution area in Russia – in Lappeenranta in 2005 and 2010, Varkaus 2008, and Kuopio 2010, were always *I. ricinus* (Publication III; Figure 13; our unpublished observations). In 2008 and 2009 *I. persulcatus* was found in Ilomantsi (Mekrijärvi) and one *I. persulcatus* female was detected in Kuhmo (63°57´ N, 29°43´ E) in 2009 (Figure 13) [Bugmyrin *et al.,* unpublished].

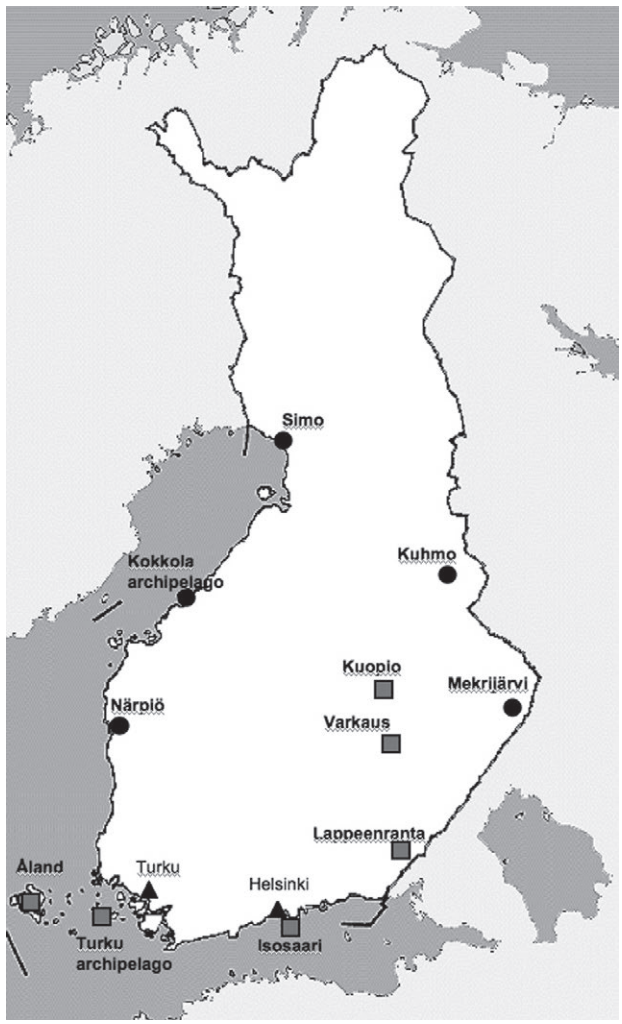

Figure 13. Observations of *I. ricinus* (gray squares) and *I. persulcatus* (black circles) in Finland since 2003. Kuhmo and Mekrijärvi, observations of *I. persulcatus* in 2008 and 2009 [Bugmyrin *et al.,* unpublished].

Öhman studied tick abundance in Finland in the 1950s (Figure 9A) [Öhman, 1961] and Brummer-Korvenkontio and others in the Åland Islands and in southeastern Finland close to the Russian border in 1950s and 1960s [Brummer-Korvenkontio *et al.,* 1973]. They found exclusively *I. ricinus*. However, Öhman studied the abundance of ticks in Finland in general by sending questionnaires to veterinarians and other enthusiastic friends of nature, and as *I. ricinus* and *I. persulcatus* look very similar with bare eyes, *I. persulcatus* may have been misinterpreted as *I. ricinus* by amateurs e.g. in the archipelago of Kokkola, where we later (2004) found *I. persulcatus* (Publication II).

In addition to *I. ricinus* and our findings of *I. persulcatus* in the archipelago of Kokkola (Publication II) and other sites along the western coast of Finland (Publcations III and IV), there are reports on several tick species from Finland: *I. trianguliceps (= I. nivalis* Rondelli), *I. lividus* Koch *(= I. plumbeus* Leach), *I. arboricola, I. uriae (= Ceratixodes uriae* Schulze), and *Hyalomma marginatum* [Saikku and Brummer-Korvenkontio, 1975; Jaenson *et al.,* 1994]. *I. frontalis* was once observed on an *Anthus trivialis* migratory bird arriving Finland in spring 2005 and *Rhipicephalus sanguineus* has been repeatedly found in dogs [Laakkonen *et al.,* 2009]. In the spring 1962 Nuorteva and Hoogstraal studied ticks on 2619 migrating birds arriving to Finland and found 99 *I. ricinus* and 11 *H. marginatum,* and 11 *I. lividus (I. plumbeus)* on breeding birds, but not a single *I. persulcatus* [Nuorteva and Hoogstraal, 1963]. Saikku and others collected ticks from birds in the 1960s, and found 252 *I. ricinus,* one *I. arboricola* and six *Hyalomma* spp. ticks [Saikku *et al.,* 1971] – still no *I. persulcatus*. Many of the "exotic" tick species found in birds are apparently merely incidental introductions to Finland. There are no previous reports of *I. persulcatus* from Finland or from the other Nordic countries, except for an engorged nymph once on a willow warbler *(Phylloscopus trochilus)* in Haparanda at the Finnish border in northeastern Sweden, in May 1992 [Jaenson *et al.,* 1994]. The nymph has been thought to have traveled on the bird when it had migrated to Sweden for the summer. Haparanda (65°50´N, 24°09´ E) is about 50 km from Simo (65°40´ N, 24°54´ E), where we found *I. persulcatus* questing and established in nature (Publication IV), and possibly *I. persulcatus* is actually distributed all over the northern and eastern coast of the Gulf of Bothnia of the Baltic Sea.

I. persulcatus ticks seem to survive in colder environments than *I. ricinus* [Hasle, 2011]. The latter is found in southern, central and coastal areas of Sweden where the mean duration of yearly snow cover is ≤ 150 days and where the growing season (daily mean temperature continuously ≥ 5°C) lasts for ≥ 160 days in a year, and in a narrow strip in the northeastern coast [Jaenson *et al.,* 2009]. In Finland we have both tick species. The northernmost place studied in this thesis was Simo, where the yearly growing season is only about 140 days [Finnish Meteorological Institute, 2010], thus probably too short for *I. ricinus*, but obviously not for *I. persulcatus*. However, other factors such as rainfall, snow cover or temperatures outside the vegetation period may affect the survival of tick species as well.

I. persulcatus on the western coast of Finland – why and when?

As mentioned before, we were the first to find *I. persulcatus* living in Finland, along the western coast. Southern, central and even south-eastern parts of Finland are inhabited by *I. ricinus* [Öhman, 1961; Brummer-Korvenkontio *et al.,* 1973; Jääskeläinen *et al.,* 2010]. However, extensive studies on tick distribution in Finland have not been done since the 1950s and possibly *I. persulcatus* lives unnoticed in the inner parts of the country as well. Actually, recent studies by

Bugmyrin and others indicate that *I. persulcatus* might well be found in the central-northern parts of the country (Figre 13), although the report of a single adult *I. persulcatus* in Kuhmo [Bugmyrin *et al.,* unpublished] does not prove a viable colony has been established there.

Ticks do not move long distances by themselves but as they may feed on a vertebrate host for days, they can be transmitted by their hosts, birds and mammals. Migratory birds carry ticks for especially long distances and over geographical barriers. Speculations that *I. persulcatus* has been introduced to the western coast of Finland by migratory birds seem justified. To Norway, tens of millions of birds migrate each year, carrying 0.15 nymphs per bird, thus introducing millions (4-13 millions) of nymphs every year [Hasle *et al.,* 2009; Hasle, 2011]. However, this is still minor compared to the amount of ticks already existing in Norwegian nature [Hasle *et al.,* 2009]. Birds transmit mostly immature stages of ticks and a great majority of them die before they reach mature stages and become capable of mating [Randolph, 1998], thus birds' impact on introducing adult ticks is only limited [Hasle, 2011]. *I. pacificus* can mate with *I. scapularis* but the offspring are sterile [Oliver *et al.,* 1993] thus if only a small number of adult *I. pacificus* were introduced to an area with *I. scapularis* already existing, they would most likely mate with more numerous *I. scapularis* and dilute off, and vice versa [Hasle, 2011]. There are no experimental data on whether *I. ricinus* and *I. persulcatus* are capable of breeding sterile offspring, thus we do not know if the same fate would face *I. persulcatus* when introduced to *I. ricinus* infested areas.

Even if the birds' role in establishing new tick populations in areas already populated by ticks is rather irrelevant [Hasle *et al.,* 2009], when new areas e.g. due to climate change or changes in land usage generate suitable habitats for ticks, birds might well be important in widening ticks distribution [Hasle *et al.,* 2009]. In western Finland land rises from the sea at the speed of about 8-9 mm per year [Mäkinen, 2009]. The Kokkola archipelago has thus appeared from the sea only a few hundred years ago.

However, *I. persulcatus* has never been observed on a bird migrating to Finland, nor other Nordic countries, except for one nymph in Haparanda, Sweden, 1992 [Jaenson *et al.,* 1994]. Furthermore, 11 TBEV-Sib strains isolated from *I. persulcatus* from Kokkola archipelago in 2004 were monophyletic and very closely related to each other (Publication II), which indicates a single event introducing TBEV to the area. This supports, although does not prove, *I. persulcatus* being transported to Kokkola archipelago once; at least there is no evidence of repeatedly brought new TBEV strains to the area. A molecular clock for TBEV strains has been developed to estimate the time of divergence [Uzcátegui *et al.,* unpublished] and with more sequence data it would be possible to estimate time frames for the beginning of radiation of the Kokkola strains and thus hypothetize when this introduction has happened.

An alternative, more romantic hypothesis could be that *I. persulcatus* was actually introduced to Kokkola archipelago by cattle evacuated from Karelia in eastern Finland during the World War II when Finland had to make over land to

Soviet Union [Öhman, 1961]. Karelian people, and their cattle, were evacuated twice: in autumn 1939, and again after their return in autumn 1944. *I. persulcatus* and TBEV-Sib are found in this area (Publication III), nowadays Russian Karelia. To our knowledge there are no reports of *I. persulcatus* in the eastern coast of Sweden, which supports the hypothesis that ticks have been transmitted mostly within Finnish borders, possible e.g. by evacuated cattle or horses used in the war. Again, *I. persulcatus* is not (at least not yet) found in continental Finland although cattle was obviously relocated there as well, possibly due to already existing *I. ricinus,* or alternatively, *I. persulcatus* lives unnoticed there because the ecological and climatic factors have not supported TBEV circulation.

Vector-switching in Simo

TBEV-Eur is usually transmitted by *I. ricinus* ticks and TBEV-Sib and TBEV-FE by *I. persulcatus* ticks [Mavtchoutko *et al.,* 2000; Lundkvist *et al.,* 2001; Charrel *et al.,* 2004]. TBEV-Eur has been occasionally detected in other tick species as well, including *I. nipponensis, H. longicornis, H. flava,* and *H. japonica* as far east as in South Korea [Kim *et al.,* 2008, 2009]. TBEV grows in several tick cell cultures *in vitro (I. scapularis, Hyalomma, Rhipicephalus, Ornithodoros)* [Ruzek *et al.,* 2008]. Süss lists several tick species that can marginally transmit TBEV-Eur: *I. hexagonus, I. abricola, H. punctata, D. marginatus* and *D. reticulatus* [Süss, 2003]. In addition, TBEV-FE has been isolated from *I. ovatus* in Japan [Süss, 2003] and TBEV-FE strain Crimea was isolated from *I. ricinus* in Ukraine [Ecker *et al.,* 1999]. Recently, TBEV-Eur isolates have been detected in *I. persulcatus* in Central Russia [Süss, 2011]. Furthermore, switching of the virus subtypes between the vectors has been observed in Latvia [Süss *et al.,* 2002], south-western Russia [Motuzova *et al.,* 2011] and in Estonia, Latvia and eastern Poland [Katargina *et al.,* 2011]. It is obvious that the traditional statement of TBEV-Eur association with *I. ricinus* and TBEV-Sib and TBEV-FE with *I. persulcatus* should be reconsidered at least in areas where *I. ricinus* and *I. persulcatus* and several TBEV subtypes overlap. Our findings of TBEV-Eur in *I. persulcatus* – and in rodents – in Simo are from an area with no evidence of co-circulation of tick species or TBEV subtypes. This indicates different dispersal routes of the virus and its vector. *I. persulcatus* may have extended its distribution northwards from Kokkola archipelago when something, possibly changing climate, has allowed its establishment in new areas. However, *I. persulcatus* in Kokkola archipelago carries TBEV-Sib subtype, and the nearest known TBEV-Eur endemic areas to Simo are in southern Finland. Explaining the combination of TBEV-Eur and *I. persulcatus* in Simo remains a challenge.

Distribution of TBE foci in Finland

The number of TBE cases has increased in Finland (Figure 10) like in many other European countries, but we still have only tens of cases yearly in the whole country. New foci have appeared since the 1960s (Figure 9B). In Finland, TBE is endemic very focally and it seems that a given focus can be very small, e.g. only one island in the archipelago like Isosaari island in the archipelago of Helsinki. All known TBE-endemic foci in the country are either in islands or peninsulas in the sea or lakes. The "hydrophilic" nature of TBE in Finland is probably based on (micro)climatic factors the surrounding water contributes to. The foci are restricted by geographical barriers, either water, or water and major road (in Simo) or railroad (in Lappeenranta). Such barriers limit the escape routes of small mammals such as rodents, the principal blood meal sources for *I. ricinus* and *I. persulcatus* immature stages, and the competent TBEV transmission hosts. The concentration of rodents carrying TBEV-infected and non-infected ticks due to barriers they cannot easily cross probably explains the very limited focality of TBE in islands and peninsulas in Finland. It seems likely that TBEV strains have been or are repeatedly brought to new areas in Finland e.g. by birds or by large mammals carrying infected ticks but the ecological, geographical, and (micro) climatic factors allow circulation and establishment of TBEV only in certain limited places. On the other hand, not all TBE-endemic foci are necessarily known. We have only looked for TBEV in regions where human cases are reported, and because TBEV enzootically circulates mostly between ticks and small mammals in nature, the known clinical human cases are likely only the tip of an iceberg.

Concluding remarks

Acute TBE is diagnosed by detecting specific IgM antibodies to TBEV. Recombinant antigens circumvent the need of infectious hazardous virus for diagnostics. This thesis describes the development of a diagnostic EIA-test based on a recombinant TBEV prME-antigen produced in insect cells. The test proved to be suitable for diagnosis of acute TBE and is now in routine use. The same antigen could possibly be applied for immunochromatographic rapid IgM test format or commercial EIA test if its production could be scaled up.

TBEV can theoretically exist in a vast area throughout the Eurasian continent where its vector tick species, *I. ricinus* and *I. persulcatus,* are found. However, due to complex ecological and climatic factors, TBEV only circulates in certain foci within the vector tick species' distribution region. Finland is located in the northernmost area of that region, and TBE is endemic only in a few small foci associated with the coast or big lakes. The endemic areas may change with predicted climate change if new areas become suitable for TBEV circulation. Several previously unnoticed TBE-endemic foci have appeared in Finland in the 2000s.

We found both the European and the Siberian TBEV subtypes circulating in Finland. A serological test for differentiating TBEV subtypes is needed. When it becomes available, the rare situation of having different virus subtypes endemic within one country with a rather uniform health care system could be utilized in studies of the speculated differences in pathogenicities to humans of the virus subtypes.

We found *I. persulcatus* transmitting the European subtype of TBEV in an isolated focus with no apparent co-circulation of several tick species nor virus subtypes. The scientific community should reconsider the traditional strict division of *I. ricinus* associated with the European and *I. persulcatus* with the two other subtypes of the TBE virus.

Both tick species relevant for TBEV transmission are found in Finland but in different areas. The overall tick distribution in the country is largerly unknown and data from the 1950s are outdated. The true northern limit of ticks in Finland, as well as the *I. ricinus* and *I. persulcatus* distributions still remain to be studied. Climate parameters and satellite modeling should be involved in mapping the tick distributions and for epidemiology studies of not only TBE but other tick-borne diseases as well. Finding of *I. persulcatus* in Finland suggests the possibility that TBE and other tick-transmitted diseases may become endemic in locations with colder climate where *I. ricinus* does not survive or where it is not capable of transmitting TBEV.

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Original publications